Major thesis

Evaluation of the performance of turbot (*Psetta maxima* L.) in RAS with and without an Upflow Sludge Blanket reactor.

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AFI-80436

Date: 2009-01-12

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Abstract

Aquaculture is the food production sector with higher development in the past years. This evolution must be based on economical and environmental sustainability. This work was part of an European project with the aim to understand the growth retardation in turbot cultured in RAS. For this, two different RAS were used, with (N=3) and without (N=3) an USB denitrifying reactor. This experiment, showed better growth rates, feed conversion, lower energy requirement for maintenance and higher retentions for fish reared under the denitrification reactor. It also showed, that this type of reactor reduces water exchange and nitrogen concentration in the water discharge, pointing to a environmentally sustainable development of the aquaculture sector, without affecting its competitiveness.

Acknowledgement

My first "obrigado" goes to my supervisors. Ep, thank you for letting me realize my thesis on recirculating aquaculture on Wageningen University, and for all the insights that you gave me. Leon, what can I say? You helped me during this last six months, getting you hands dirty with us, and I did not forget the 4 am shift! No words can explain your help during the experiment and the writing of the report. For all your help, obrigado!

Tinno and Ronald, thank you for you patience teaching more than once how to use the lab material. Tinno keep on listening to good music, that is the spirit! Ronald, some times you are to much worried with lab rules, but I understand that accidents can not happen.

Thierry, now is my time to say: I need a vacations! Thank you, without you this would be far more complicated, you helped me a lot. I hope that I can retribute you some day.

Mascha and Niccoló, thank you for your help with the statistical analysis. It was precious!

Elisabete e Filipe obrigado pela revisão do inglês, ajudou muito tornando o relatório mais compreensível.

Aos meus pais, meus irmãos (especialmente ao grande Xerife Pepe Legal) e à Inês*, obrigado pelo apoio nos momentos mais difíceis, sei que se pudessem teriam ajudado mais!

Malta GEAQUA e amigos de infância, obrigado pelo apoio e por não me desencaminharem mais do que eu já me desencaminho!

To all: dank wel!

List of abbreviations

ADC	apparent digestibility coefficient
AIA	acid insoluble ash
BW	body weight
BUE	branchial urinary energy
C	carbon
CF	crude fat
COD	chemical oxygen demand
CP	crude protein
DE	digestible energy
DM	dry matter
FAO	Food and Agriculture organization of the United Nations
FCR	feed conversion ratio
FE	fecal energy
FI	feed intake
G	growth
GE	growth energy
GIF	Growth Inhibition Factor
GRRAS	Growth Retardation in Recirculating Aquaculture Systems
H	heat
LC50	lethal concentration to 50% of the population
LOEC	lowest observable effect concentration
ME	metabolize energy
MEm	energy requirement for maintenance
MBW	mean metabolic body weight
N	nitrogen
N_2	nitrogen gas
NH_3	ammonium
NH_4	ammonia
NO	nitric oxide
NO_2	nitrite
P	phosphorus
ppt	parts per thousand
RAS	recirculating aquaculture system
RE	retained energy
RFR	relative feeding rate
RFRm	relative feeding rate of metabolic weight
RGRm	relative growth rate of mean metabolic body weight
RP	retained protein
S.D.	standard deviation
SGR	specific growth rate
TAN	total ammonium nitrogen
TSS	total suspend solids
USB	upflow sludge blanket
UV	ultra violet
VFA	volatile fatty acids
VSS	volatile suspended solids

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Introduction

World aquaculture has grown tremendously during the past fifty years from a production of less than 1 million ton in the early 1950s to 58,4 million tonnes by 2004 (FAO, 2006) corresponding to almost 50% of the world's consumed fish. Based on FAO's (2006) projections, to maintain the per capita consumption of fish for 2030, it will be necessary at least an additional 40 million tonnes of nowadays production.

It is widely acknowledged that fish supplies from the world fisheries are unlikely to increase and the expansion of the aquaculture sector will provide the solution for the problem, but with the increasingly limited access to costal areas due to tourism and recreational activities, pressure is put on the aquaculture sector to develop towards a higher sustainability, both economic and environmental.

Recirculating Aquaculture System (RAS) are less susceptible to sudden environmental changes since most of the water flow is reused, minimizing any potential harmful impact. Furthermore, RAS is more environmentally sustainable than other methods, reducing water consumption, conserves energy, allows better control over environmental parameters and is basically site independent. (Labatut and Olivares, 2004).

Turbot production is one of the most established in saltwater recirculation systems, although exists a lack of knowledge in those systems, regarding growth rates. It is known that growth rate in recirculating systems is inferior when compared to growth in flow-through systems, ranging from 15% to 20% less in RAS. The GRRAS (Towards Elimination of Growth Retardation in Marine Recirculating Aquaculture Systems for Turbot) project is funded by the European Union with the aim of evaluating the cause of growth retardation of turbot in recirculating aquaculture systems.

With the increase in water reuse, accumulation of substances came along. Among this substances, nitrate the end product of nitrification seems to be the most abundant, but other like toxic metals, or pheromones released by fish are also likely to accumulate. All this compounds produced either by fish or bacteria, that cause growth retardation, are called Growth Inhibition Factors (GIF).

The of denitrification is being more frequently used as a way to remove nitrate from the water, reducing the water discharge and the nitrogen compounds in it, applied has one type of biofiltration. But others studies are point on the directions that this denitrifying

bacteria have the ability to produce GIF neutralizing substances. It is therefore, important to understand the effect of this type of filtration in the performance of fish.

This study was conducted to evaluate the growth performance of turbot reared with and without an Upflow Sludge Blanket denitrifying reactor (USB), with two different water exchanges. For the control treatment, without USB was established a flow rate of 300 L/kg of feed, while the treatment with USB, it was indented to establish a water exchange of 30 L/kg of feed. Both treatment had the concentration of nitrate, 150 mg/L

1. Literature review

1.1 *Psetta maxima* [(Linnaeus, 1758)](#): General overview

Turbot (*Psetta maxima* Linnaeus, 1758) is a left eyed flatfish with asymmetric and almost round body. On the eye side it presents bony protuberances randomly distributed with variable color, the opposite side has whitish color (www.fao.org), although cultured fish often show variable pigmentation on the blind side. They are commonly found in waters from the Mediterranean throughout the European coast until the Arctic Circle. It is a benthic marine fish living in depth ranges from 10 to 70 m, usually in sandy, rocky, muddy or mixed bottom in inshore waters (www.fishbase.org).

It is a carnivorous active predator species, with juveniles feeding on molluscs and crustaceans, and adults mainly on fish and cephalopods. Spawning (sequenced, every 2-4 days) usually takes place between February and April in the Mediterranean, and between May and July in the Atlantic (www.fao.org).

Under culture conditions, turbot reaches sexual maturity at an age of about 24 months, and this does not affect growth due to the small size of the gonads by that time (Cal, Vidal et al. 2006). Females are usually larger and reach sexual maturity normally later than males.

The life cycle of turbot is characterized by its extraordinary metamorphosis during the larvae stage, transforming from a bilaterally symmetrical body to an asymmetrical body shape, associated with a life style change from pelagic to benthic habits.

1.2 Fisheries, Production & Market

With some marked oscillations during the 1980s, turbot catches yield more than 7 000 tones annually. In the last years a considerable proportion of total production derives from aquaculture, mainly in Spain and France, with the wild catches of turbot declining significantly over the last 20 years or so (APROMAR, 2008).

Farmed turbot production is very recent in Europe but is growing rapidly. For the last few years, the European production has remained stable, reaching 7.6 Tons in 2007, which is 6.3% higher than the production in 2006 and with a prediction to grow 20.8% more in 2008 (APROMAR, 2008). Spanish annual turbot production has practically doubled since 1998, corresponding to 75.9% of the total European production in 2002 and 77.9% in 2007,

followed by France with 10.2% and Portugal with 7.1% in the same year (APROMAR, 2008). The price has remained stable for the last 5 years, going around 9€/Kg (price of first sell), recently with the world economic crisis the prices have been decreasing around 3€/Kg, reaching 6 to 7€/Kg at first sell in Spain (La Opinión Coruña, 2008).

In the early stages of the industry, turbot were produced in inshore flow-through systems. Increasingly these are being replaced by partially closed and closed recirculation systems. Figure 1, shows briefly the stages of turbot production.
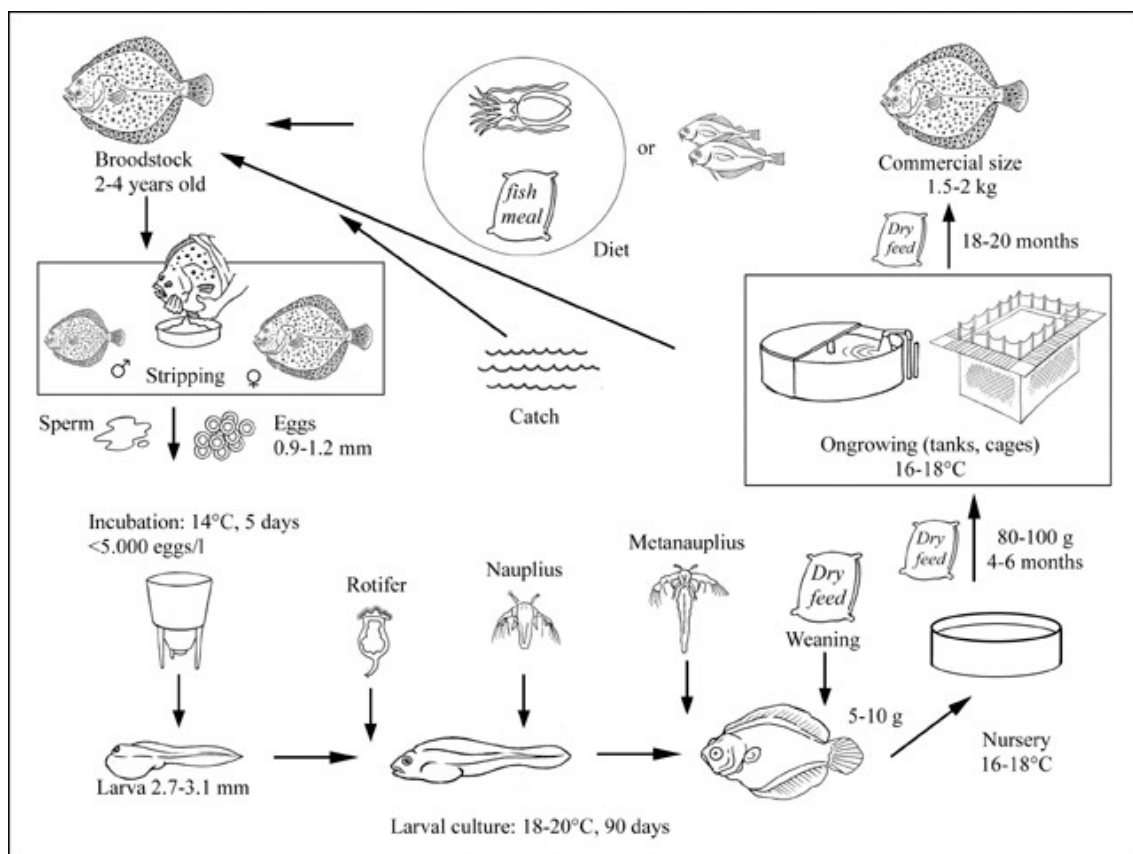
Fig. 1: Production cycle of turbot, showing different stages of production form broodstock management to ongrowing techniques (www.fao.org).

The accessibility to coastal areas is increasingly limited as tourism and recreational activities develop, also environmental issues such as nutrient emissions to aquatic ecosystems are forcing aquaculture to move towards land-based farming, with the technology to reuse water (Aubin, Papatryphon et al. 2006).

In recirculation systems, the outlet water from fish tanks is re-used after the appropriate treatment, reducing the amount of new water added. Being the water treatment units the mainly difference from other systems.

Recirculating Aquaculture System (RAS) can be defined as an aquaculture system that incorporates treatment and reuse of water, with less water waste per day and different treatments being applied for different systems. For instance, different types of trickling filters, different systems for solids settlement, or Ozone versus U.V. filtration usage depends on the final intent.

Due to this, RAS are less susceptible to sudden environmental changes since most of the water parameters are controlled, minimizing any potential harmful impact. Furthermore, RAS is more environmentally sustainable than other methods, reduces water consumption, conserves energy, allows better control over environmental parameters and is basically site independent (Labatut and Olivares, 2004).

Although RAS have advantages, they also present disadvantages. The main ones are investment and operating costs, mostly due to number and technology of water treatments. Also the reuse of water causes accumulation of substances in the water, substances like nitrate (the end product of nitrification), organic compounds, heavy metals among others, are considered to affect growth performance of fish. This subject will be discussed further in more detail.

1.3 Growth

Growth in teleost fishes occurs according to characteristics steps for each species being affected by several factors (Boeuf, Boujard et al. 1999) with the increase of wet weight as final result. This increase in wet weight can be described bioenergetically, as being the sum of energy for metabolism, the energy lost in metabolism (consumption of oxygen and production of heat and carbon dioxide a.o.), energy lost in faeces and energy for storage and/or growth. When the energy intake by external sources is higher than the energy needed for maintenance (sufficient energy to maintain basic metabolic activity) fish will grow (Jobling, M. 1994).

There are several reasons why growth in fish is different from that in birds and mammals. The fact of being poikilothermic and living in a supportive medium reduce the energy needed for thermoregulation and overcoming gravity, which can be used for growth (Boeuf, Boujard et al. 1999). Growth rate is dependent on water quality, food availability and

developmental stage (Sumpter, 1992). Beside the rate that the animal grows, but also is important the energetic efficiency which the animal grows (Fraser, Rogers et al. 2007).

Growth is primarily achieved by the synthesis and retention of proteins; all the proteins within the individual (protein pool) are characterized by three inter-related processes: protein syntheses (proteins constantly entering the protein pool), protein retention and protein degradation (proteins being removed from the protein pool). With the retention of protein, water deposition comes along, being protein growth the major factor to increase body weight (Jobling, M. 1994).

So, growth in fish can be resumed to the retention of a portion of the synthesized protein transforming in soft tissue growth (Fraser, Rogers et al. 2007).

1.3.1 Factors controlling somatic growth

As referred before, growth occurs when the energy intake is higher than the energy needed for maintenance, as a result any factor that affects rates of food consumption and metabolism has an effect on growth. These factors can be either internal or external (Jobling, M. 1994).

1.3.2 Internal Factors

Boeuf and Boujard et al (1999) differentiated the internal factors in three axis: Thyrotropic, Somatotropic and Gonadotropic axis.

1.3.2.1 Thyrotropic axis

The thyroid hormones are known to influence early development and growth (Boeuf, Boujard et al. 1999). The control of releasing hormones (thyroid hormone) by the thyroid gland takes place on the brain-pituitary axis. Also Iodide (I-), a scarce environmental element, is transported from the plasma into thyroid follicles which synthesize and store thyroid hormone.

This thyroid hormone, has his greater importance in the early live stages of fish, especially in flatfish due to their unique transformation from bilaterally symmetrical body

shape life to asymmetrical body shape and benthic life, with all the alterations of anatomy and muscle distribution.

Studies reffered in Mommsen, Moon et al. (2001), showed that fishes treated with thyroid hormones not only had better growth performance, but also increase in appetite and amount of amino acids in plasma.

1.3.2.2 Somatotropic axis

Growth hormone is a polypeptide produced and released by the somatotrophic cells located in the pituitary, this releasing is quite complex in teleost fish, since different hormones controls this release for different species. For example, in catfish and tilapia the release of growth hormone is stimulated by Growth Hormone-releasing Hormone, and in goldfish and carp the release is stimulated by Gonadotropin-releasing Hormone, but none of these two seems to stimulate the releasing in European eel (Rousseau, Le Belle et al. 2001).

Due to this lack of knowledge, intensive research has occurred during the last decades and it is known that growth hormone is an essential regulator of growth and can change the rate of muscle protein synthesis by a number of indirect routes, but also plays an important role on osmoregulatory, reproduction and immune system (Bjornsson, Johansson, et al., 2003).

Growth hormone also plays a role in development of skeletal tissues controlling Insulin-like Growth factor (IGF) release. IGF is known to improve growth in bone and cartilaginous tissues. Recent studies showed novel functions of IGF system, on osmoregulation, reproduction and regulation of embryonic and larval development (Wood, Duan et al. 2005).

1.3.2.3 Gonadotropic axis

Somatic and reproductive growth can be viewed as competitors of the same limited source, this is supported by the drop in somatic growth rate while fish start gonadal maturation. It has been proved that fish reduces somatic growth to be able to spend more energy in gonadal growth (Jobling, M. 1994).

Apart from growth hormone and IGFs, other hormones like steroids or gonadotropic-releasing hormone play an important role in gonadal growth. The releasing of

such hormones must be synchronized with the spawning season, which can be regulated by the quantity of melatonin segregated by the pineal gland, creating an inside calendar for the fish.

1.3.3 External Factors

The same authors (Boeuf, Boujard et al. 1999) have differentiated the external factors in determining factors and limiting factors. Determining factors such as temperature, salinity and photoperiod act directly on the receptors to increase or decrease growth, on the other hand limiting factors define a specific threshold for example levels of oxygen or ammonia or creating a tolerance range like pH.

Due to the species subject of the experiment, these factors will be analyzed mainly for turbot.

1.3.3.1 Temperature

Temperature is known to influence rates of ingestion, metabolism and growth (Jobling, M. 1994). Growth rate increases with the increase of temperature, but a decline in growth at high temperatures is observed. On the other hand, the routine metabolism rate does not decline, this will provoke a higher need of food for maintenance or for basic metabolic activity. So depending on the availability of food, fish can have better growth rates at higher or lower temperatures. For instance in a restricted feed regime, fish will grow better at lower temperatures due to their lower basic metabolic activity.

Fish, as ectotherms, have their body temperature closely related to environment, consequently higher metabolism at higher temperatures. Different studies have been made to determine the optimum growth rate related to temperature, although caution must be taken with the experimental design since, as referred above, the amount of feed related with temperature can influence the growth rate.

Boeuf et al (1999) refers, based on other studies, that the optimum temperature range for growth in turbot is 16-19°C, a decrease in growth with temperatures below 14°C is observed. Imsland et al (2001), showed differences in growth performance being partly due to different temperature adaptations, when he studied turbot populations from different latitudes (Norway, Scotland and France). His study showed that growth performance was higher for all

populations at temperatures between 18 and 22°C (at 15‰), but the highest growth performance was observed in the Norwegian population. This higher growth can be related to a shorter growing season (corresponding to a season with higher temperature), acting like a compensatory mechanism.

The above values seem to be, among literature reviewed, the most common values of temperature for optimum growth. In the existing literature, the references are for larvae and juveniles which make it the optimum temperature for growth rate overestimated.

The results showed an optimum temperature ranging from 16° to 22° C, being temperature an important factor on growth. However it is pointed out in these studies that important interaction with other factors occur, influencing growth rate.

1.3.3.2 Salinity

Fish spend energy in ionic and osmotic regulation, since the surrounding medium has a different concentration from those of the body fluids (Jobling, M. 1994). Salinity acts at the level of the brain pituitary which, by hormonal stimuli, releases other hormones with osmoregulatory functions like growth hormone. These interactions may explain implication of salinity in growth.

There is some evidence that turbot, like other marine fish, uses estuary has a nursery ground for juveniles, which makes them tolerant to changes in water salinity. Studies showed better growth rates at 10‰ -19 ‰, comparing with fish in full strength seawater. (Gaumet, Boeuf et al, 1995)

On other study, carried by Imsland et al. (2001), growth, food consumption, daily feed intake and feed conversion efficiency were highest at 15‰ and lowest at 33.5‰ in fish reared at the temperatures 18ºC and 22ºC. Their study showed that the capability of turbot to adapt to different temperatures and salinities are correlated, and the improvement of growth can be achieved by rearing turbot at lower salinities than full strength seawater (Imsland, Foss et al. 2001).

1.3.3.3 Light

Photoperiod is known to influence both feeding and growth in fish. Light stimulates the photoreceptors in the pineal gland and retina, to release melatonin, a neurohormonal signal that will transport a message to the brain.

Melatonin is a time-keeping molecule that is released rhythmically during the night, qualifying not only daily time but also calendar time by altering the duration and amplitude of secretion with photoperiod and temperature, respectively (Confente, El M'Rabet et al. 2008). This hormone is known to be involved in several physiological processes, such as feeding, growth, development, metamorphosis and especially reproduction.

An experiment made by Mallekh et al (1998) to understand the influence of environmental factors on turbot growth, showed that day length had significant influence on appetite of turbot, increasing it when the light hours were longer than 15 h. Although this experiment was made in ponds, which could mean that longer photoperiod are related to higher temperatures increasing metabolism and consequently growth.

1.3.3.4 Oxygen

The metabolic rates of well-fed fish are higher than those of starving fish, and oxygen consumption increases with the increase of feeding rate. This is due to oxygen cellular function, used to burn proteins, fat and carbohydrates delivering energy for swimming, osmotic regulation, growth, among others. When oxygen intake is not enough to sustain cellular metabolism, anaerobic metabolic pathways start to take place in ATP production, with higher energetic costs (Jobling, M. 1994) this is a temporary process until an oxygen debt is built.

Due to turbot behavior (benthic species), the requirements of oxygen are lower than for pelagic species that require higher amounts of oxygen for continuously swimming, also due to physiological gill surface area of flatfish being limited compared with pelagic fish, metabolic rate for benthic species is lower (Mallekh and Lagardére, 2002)

Person-le, Ruyet et al (2002), concluded that turbot can adapt easily to moderate hyperoxic conditions, with a lack of improvement on growth showed that the interest O_2 supplementation is to maintain water concentration near air saturation level, but an oxygen shortage will affect growth metabolism. Mallekh and Lagardére (2002) stated that, under

farming conditions oxygen supersaturations adds nothing to turbot appetite and due to economic reasons should be avoided.

On other experiment, Pichavant et al (2000) studied the influence of hypoxia on growth of juvenile turbot. This study showed that, feed intake and growth were significantly lower at oxygen concentration below 5,0 mg/L (hypoxia), this was interpreted as energy-saving strategy thus decreasing feed intake the oxygen need to burn down feed is lower.

1.3.3.5 Carbon dioxide

Carbon dioxide has been used as an anesthetic, making it is easy to understand that this gas has the ability to disrupt the normal physiological functions of fish (Jobling, M. 1994).

Carbon dioxide is considered a limiting factor, as fish can not excrete carbon dioxide when the environmental concentration is too high, causing fall in blood pH. Increase of carbon dioxide concentration is linearly related with growth reduction in fish, showing calcareous deposits in the kidneys.

1.3.3.6 Total Ammonia Nitrogen

Fish are able to utilize dietary protein very efficiently. Despite the fact that they use a significant portion of digestible protein for energetic purposes, they produce large quantities of nitrogenous metabolites. The main end product of nitrogen metabolism in teleost fish is ammonia (Dosdat, Servais et al. 1996).

Accumulation of this nitrogen compounds in water culture is a very critical point due to their toxicity. The process of ammonia removal, in recirculation system, is normally by biological filtration where nitrification occurs (oxidation of ammonia to nitrite and finally nitrate) (Timmons and Ebeling, 2007).

Ammonia is mainly excreted by the gills, and in solution it exists as unionized NH_3-N or positively charged NH_4^+-N and the sum of them comprises the total ammonia nitrogen (TAN) representing the inorganic nitrogen compounds in terms of the nitrogen they contain. Due to their dissociation constant more than 95% of TAN in water exists as NH_4^+.

A study made by Dosdat, Seviras et al (1996), compared the nitrogen utilisation and excretion of five fish species, among them turbot. They were raised under similar feeding and environmental conditions, turbot showed the lowest ammonia excretion rate (20% of ingested nitrogen) due to his high protein efficiency.

Other study, designed to check the chronic effect of ammonia in juvenile turbot (14 to 104 g) showed that growth stopped at 0,8 mg unionised ammonia nitrogen (UIA-N)/l, they also observed that bigger fish had less tolerance to ammonia, for fish with 104 g the LOEC (lowest-observable-effect concentration) was 0,10 mg UIA-N/l and for smaller fish values were 0,41 and 0,21 mg UIA/l for 14 and 23 g turbot, respectively (Ruyet, Galland et al. 1997).

The proportion of TAN in the water is influenced by pH and temperature, being the fluctuations of pH the factor with higher influence on TAN. The increase in pH leads to a higher concentration of UIA, whereas a lower pH decreases its level (Ip, Chew et al. 2001).

1.3.3.7 Nitrite

Nitrite is an intermediate and important product in bacterial nitrification and denitrification processes in the nitrogen cycle. It is converted to nitrate as quickly as it is produced, and is constantly based produced as an intermediary step (Timmons and Ebeling, 2007).

The blood appears to be the primary target of nitrite action, oxidizing the iron in the haemoglobin molecule resulting in methaemoglobin. The methaemoglobin reduces the total oxygen-carrying capacity of the blood (Kroupova, Machova et al, 2005).

There are few studies regarding to limit levels of nitrite for flatfishes, but Huguenin and Colt (2002) suggested 0,1 mg/l has the threshold for aquatic animals. Although, nitrite toxicity to fish depends on external and internal factors, such as salinity, length of exposure, pH, temperature, fish size and age, but the most important is water chemistry, especially chloride concentration, increasing toxicity with increasing chloride concentration in water (Kroupova, Machova et al, 2005).

1.3.3.8 Nitrate

Nitrate is the end-product of nitrification and is the least toxic of the nitrogenous compounds, due to this usually nitrate concentrations are higher than other nitrogenous compounds.

The reason why nitrate is not so toxic to fish as the other compounds is due to a low branchial permeability of it, being much higher the uptake of ammonium and nitrite. Although, nitrate affects the transportation of oxygen changing, like nitrite, haemoglobin into methemoglobin (Camargo, Alonso et al. 2005).

A study conducted with Medaka fish, showed that even at relatively low concentrations, nitrate can affect embryonic development, growth and egg-laying capacity. It was determined that concentration until 25 mg NO_3-N/l (110 mg/l NO_3) is considered to be safe for long-term exposure. In the same study, and in the chronic toxicity experiment, growth suppression in body weight was found at higher concentrations between 100 and 125 mg NO_3-N/l (Shimura, Ma et al, 2004).

On other study, the authors considered the proposed level of 20 mg NO3-N/l for culturing seawater animals may in general be acceptable (Camargo, Alonso et al. 2005).

These values seem to be very low, compared with the values found by Pierce, Weeks et al. (1993) although their study was focused on the acute toxicity of nitrate for five marine species (*Raja eglanteria, Trachinotus carolinus, Centropristis striata, Monacanthus hispidus, Pomacentrus leucostictus),* on trials of 96 hours. *Raja eglanteria,* a flatfish showed no acute toxicity until 960 mg/L of nitrate, and *Pomacentrus leucostictus* showed high tolerance, with a 96 h LC 50 (lethal concentration on 50% cases) greatter than 3000 mg/L. The authors referred that more studies should be done, focusing on long-therm expositure with possible implications on growth and reproduction, giving 500 mg/L of nitrate as near acute toxicity for some fish species.

A study carried out to acknowledge the influence of nitrate in release of sex steroids and other hormones with Siberian sturgeon, showed that elevated nitrate is capable of altering the steroid profiles of cultured female sturgeon, and is able to alter the secondary stress response, defined by plasma glucose concentrations (Hamlin 2006). Although it is known that this species is more sensitive to nitrate and comparison with other species must be done with caution.

Sublethal effects of nitrate include endocrine alterations which have been shown to alter metabolism, reproductive function and development (Hamlin, Moore et al. 2008).

Excess nitrate in aquaculture has traditionally been reduced by water exchange and more recently by denitrification filters (Timmons and Ebeling, 2007).

1.3.4 Mineral availability

Fish are surrounded by water that contains mineral salts in solution (in seawater the more abundant ions are: Cl^-, Na^+, Mg^{2+}, SO_4^{2-} and Ca^{2+}) and they have the ability to absorb some inorganic elements not only from their diets but also from their external environment in both freshwater and seawater. Generally Ca, Mg, Na, K, Fe, Zn, Cu, and Se are absorbed from the water to ensure the nutritional requirements of fish, but phosphate, chlorides and sulfates have to be supplemented in feed to have the right nutritional requirement (Lall and Lewis-McCrea 2007).

An experiment made with trout showed that when it was in seawater the intake of minerals increased, showing that the increase of drinking water increased the mineral intake (Timmons and Ebeling, 2007), it can be affirmed that minerals in water can be uptaken by fish increasing the amount of drinking water.

Calcium and phosphorus are closely related to the development and maintenance of the skeletal system as well as various metabolic functions (Jobling, M. 1994). The calcium requirement of fish is related in large part by their ability to absorb these ions directly from the aquatic environment. On the other hand, the concentration of P is too low in both freshwater and seawater, being diet the main source of P (Lall and Lewis-McCrea 2007).

Many essential micro or trace elements such as zinc, manganese and copper are required for growth and development of healthy bones of fish (Lall, in Lall and Lewis-McCrea 2007). Also minerals can interact with themselves, or like Selenium can interact with toxic heavy metals reducing the biological availabity of both. (Lall and Lewis-McCrea 2007)

1.3.5 Humic acids

Humic substances occur with the microbial degradation of organic matter and ultimately biomolecules like protein, lipids and carbohydrates dispersed in the environment after the death of living cells.

Microvesiles are formed by humic acids in aqueous solution (Reid et al., 1991). Therefore, it is possible that pheromonal steroids, released into the water by fish, may be dissolved by humic acid microvesicles and therefore become unavailable for chemical stimuli of fish (Hubbard et al., 2002).

Recent studies on water treatment are focusing on the removal of metals from the water, since they are not biodegradable the usage of humic acids and its absorbent properties is being targeted for heavy metals removal from water (Coles and Yong, 2006). A study made to understand the Arsenic and heavy metal mobilization from mine tailings referred that humic acids might be used to remove arsenic and heavy metals simultaneously under alkaline conditions, in fact pH is a factor that has major influence on this kind of removal (Wang and Mulligan, 2009).

Meinelt et al (2001) referred that the interaction between humic acids is more complex that it may seem, in some studies the presence of humic acids reduce the toxicity in water but in other cases it increases. For instance, the presence of humic acids, reduced the toxicity of cadmium in small rainbow trout, but tests with *Daphina sp*. showed an increase of toxicity in the presence of humic acids. Also humic acids can bind with minerals reducing the bioavalability of them, this could become a problem since it could turn into a limiting factor.

1.3.6 Other factors

Besides the parameters above described, there are more parameters that can affect growth. They will be generally approached. Parameters like density, tank design, husbandry techniques and feeding strategies can be easily controlled but other parameters like stress are more difficult to control, being directly related to environmental factors like temperature, pH, pollutants, salinity, among others.

The adverse environmental circumstances are the cause of stress in fish, and it is very difficult to establish borderlines that define when fish became under the stress because of this, levels of cortisol are generally accepted as a measurement of stress (Van Weerd and Komen 1998).

The release of cortisol is related to slowing down wounds increasing the susceptibility of pathogens (Bonga, 1997). Normally in culture conditions, stress is long-lasting and therefore non-adaptive (Van Weerd and Komen 1998). The implications of stress

are various, affecting growth by decreasing the feed intake, or absorption or even its utilization (Van Weerd and Komen 1998), affecting also the immune system and oxygen uptake. Also fish on an attempt to adapt to an adverse environmental situation, reallocate energy that usually spend for growth or reproduction to activities that restore homeostasis, such as respiration, osmoregulation and swimming (Bonga, 1997).

1.3.7 GIF: Growth Inhibition Factors

As referred before, a substantial problem in using RAS for marine aquaculture is the growth retardation. It is believed that, this growth retardation is due to accumulation of substances produced either by fish and/or bacteria, considered to be prejudicial to fish development. To these substances it is called **growth inhibition factors** (GIF). The information of the nature of the substances is limited and incomplete (GRRAS, 2006).

A study made with sea bass (Deviller et al., 2004), comparing flow-through and two types of RAS showed that, fish growth decreased in both RAS comparing with flow-through associated with a lower feed intake. The authors referred that the regression in growth could not be associated with the decrease of water quality, but they pointed has the probable cause. The same author but other study (Deviller and Palluel et al., 2005), looked for metals concentration in sea bass reared in RAS and flow-through. In this experiment, he found significant differences between treatments, being the metals concentration higher in fish cultures on RAS although the accumulation values were lower than the recommended by FAO/WHO. Both studies show that the accumulation of substances could have influence on fish development, but further studies are needed.

Roales (1980), studied the effect of growth inhibition factors on lipid content in zebrafish, his study showed a better growth of zebrafish reared with water passing through activated charcoal compared with fish without the type of filtration. The author based his theory on fact that GIF could be pheromones, Fuld (1977) (in Roales et al., (1980)) observed that crowding factor influenced the thyroid hormone, increasing the oxidation rate, thus decreasing lipid content. This explains why fish under the extracted water (activated charcoal) had higher lipid contents.

On a review of chemical communication in freshwater (Solomon, 1976), this crowding factor is considered to be chemical similar with esters, or complex linked with lipids. This crowding factor could function in nature, has a way to disperse fish reducing

competition among them. Crowding factor was also observed by Rose (1959) (in, Solomon, ,1976), where the author observed that in aquaria which was considered crowded, shortly after feeding commenced, size differences appeared. Where larger fish continue growing and smaller fish started to meager despite abundance of feed, when this fish were transfer to other aquaria they grew normally.

With the above, GIF can be grouped in different types of substances, from metals to complex organic molecules. As stated by Hara (1975) "sensory receptors are the immediate detectors of environmental stimuli" and for this subject the most important of these stimuli is the chemical either by olfactory or by taste. Since fish live in aquatic medium, to be able to sense GIF they have to be soluble in water, as it was referred above humic acids are able to bind with hormones making them insoluble in water, disabling fish to sensing it.

GIF as the name says inhibit growth, but how does it inhibits? As it was said, literature on this subject is scarce, but some hyphothesis may be elaborated. For instance, GIF can affect the feed intake, by making the surrounding environment stressful for fish, decreasing growth.

GIF could also influence the immunological system of fish and as a response, fish has to reallocate energy from used growth to overcome this adverse situation, or GIF could simply elevated the energy requirements for maintenance metabolism by affecting for instance osmoregulation, influencing the requirements of energy for maintenance metabolism.

Besides that, GIF could also be considered as signaling factor. As it is referred above, substances released influencing fish development, considered to be a way of communication between fish, showed to be similar to esters. It is known that almost every family of steroid hormone occurs in esterified form (Hochberg, Pahuja et al., 1991). With this we can say that cortisol (a corticosteroid) could be considered as GIF, as other steroids normally used in chemical communication in fish and pheromones in general, but little literature is available on this matter.

1.3.8 Feed: source for growth

Feeding fish is more complicated than feeding domestic animals, due to their aquatic life style, with implications in feed formulation and loss of un-eaten feed. Since feed is the main cost in intensive fish farming, farmers saw the need to improve the efficiency of feed. The development of feed budgets based on the daily energetic requirements of the cultured

fish, are viewed as the solution to overcome these economic losses. The utilization of bioenergetic models is becoming more and more frequently to improve efficiency of fish farming.

Bioenergetic can be simply described as the study of the balance among dietary energy intake, expenditure, and gain being able to look at the dietary component utilization by animals (Bureau, Kaushik et al. 2003).

By the model of these authors, fish obtain energy via feed, which is divided in three kinds of complex chemical compounds which contain energy: carbohydrates, lipids and proteins, being lipids the component with higher energy content and carbohydrates the lowest.

Gross energy is the total energy content of the feed. The feed consumed by the fish contains complex compounds which are decomposed into simpler components absorbed in the gastro-intestinal tract, but a part of energy will be lost with the faeces (FE) because it is indigestible, the variation of digestibility of feeds is the major factor in the usage of energy by fish.

Digestible energy (DE) is the energy which is digested and absorbed in the fish, and is calculated by subtracting the **faeces** (FE) from **gross energy** (GE). Some of this energy is excreted by the kidney and gills of fish, and this energy loss is called **branchial and urinary energy loss** (BUE), which can be estimated from the nitrogen balance.

The **metabolisable energy** (ME) is the remaining energy which is available for metabolic processes in organisms. A part of this energy is used for functions of the body necessary to maintain life, like blood circulation, pulmonary ventilation, membrane transport of ions among others, this is called energy for basal metabolism together with energy for voluntary or resting activity they form the amount of **energy for maintenance** (MEm). There are different factors that influence the amount of energy which is used for maintenance processes, among them are body weight and temperature (MEm is lost by the body as **heat, H**).

The remaining part of this energy is used for tissue deposition, growth (MEp) this deposition is also linked with energy costs, has **heat** produced. Part of the metabolisable energy taken in as diet which is not dissipated as heat is **retained** in the body as new tissue constituents (RE) it can be either positive or negative.

1.3.9 Energy requirements for maintenance

"Animals require a continuous supply of energy for those functions of the body immediately necessary for maintaining life, regardless of whether or not feed is consumed" (Bureau, Kaushik et al. 2003). This expenditure of energy is related to such as osmoregulation, blood circulation, cells activity and muscle tone. The energy requirement for maintenance (MEm), can be defined has the energy needed for "no-growth", keeping the animal in zero energy balance (Bureau, Kaushik et al. 2003). Before fish uses energy from feed to growth or reproduction, it has to cover the MEm, thus knowing this value is very important for an aquaculture production.

This data are usually obtained by dose-response experiments, with several types of feed containing different amount of nutrients being the maintenance requirement at which there is no energy gain (Peres and Oliva-Teles 2005). More recently a factorial approach is being used, with a regression of ME intake as a function of protein and lipid deposition, showing to be usueful determining partioning of ME in maintenance and growth (Azevedo et al., 2005). By this approach lipid and protein deposition are considered function of ME.

As it was said before, fish grow with protein and fat deposition, this is evaluated as k_P and k_L being efficiency of protein and fat deposition respectively. Generally, k_L is higher than k_P, since the efficiency of lipids is higher compared with the efficiency of proteins. And the efficiency of energy retained as a whole, that consists in the efficiency of protein and fat deposition is called k_g. Table 1, shows some values of MEm for different studies.

Table 1: Estimations of energy requirement for maintenance (here described as HEm); efficiency of protein deposition (k_p) and lipid deposition (here described as k_f) (from: (Bureau, Kaushik et al. 2002)

Species	HEm	K_p	K_f	Ref.
Common carp	$42 \text{ kJ kg}^{0.75} \text{ day}^{-1}$	0.56	0.72	Schwartz and Kirschgessner (1995)
European sea bass	$42 \text{ kJ kg}^{0.79} \text{ day}^{-1}$	0.54	0.91	Lupatsch (2000)
Gilthead sea bream	$59 \text{ kJ kg}^{0.83} \text{ day}^{-1}$	0.47	0.66	Lupatsch (2000)
Grouper	$35 \text{ kJ kg}^{0.83} \text{ day}^{-1}$	0.44	0.91	Lupatch (2000)
Rainbow trout	$1.37 \text{ kJ g}^{0.39} \text{ day}^{-1}$	0.54	0.90^b	Rodehutscord and Pfeffer (1999)

1.4 Denitrification

As it is referred above, only 20%-30% of the nitrogen introduced in the feed is incorporated by fish, the part non-incorporated is excreted mainly as ammonia. Nitrate is the end-product of nitrification. Due to this their concentration in the water culture is comparatively high, especially in systems operating with low water exchange and high densities (Timmos and Ebbeling, 2007).

By denitrification, micro-organisms convert nitrate into atmospheric nitrogen. This is an anaerobic process in which nitrate acts as the oxidizing agent. Denitrification is widespread in nature, and occurs in the presence of nitrate, low or non oxygen levels, denitrifying bacteria and a carbon source.

As we saw previously, nitrate is the less toxic nitrogen compound, although it has some implications in growth and can act has an endocrinological disruptor. So it has to be removed from the culture water. The main methods of nitrate removal are, by means of water exchange or using heterotrophic bacteria. The first is the more vulgarly used but not environmentally friendly the second is starting to be more frequently used and therefore more studies about this subject are appearing.

1.4.1 Bacterial community

Denitrification constitutes one of the main branches of the global nitrogen cycle sustained by bacteria, it is part of the bioenergetic equipment of the bacterial cell. Denitrification extends beyond the bacteria to the archaea, where it is found among the halophilic and hyperthermophilic branches of this kingdom and may have evolutionary significance (Zumft, 1997), also fungi were studied on their denitrification capacity.

Most denitrifiers are aerobic heterotrophic organisms that from the oxidation of a carbon source, transfer redox equivalents to an N oxide under anaerobic conditions, a wide group of bacteria being the proteobaceria the predominant group (Alpha-, Beta-, Gama-, and Epsilonproteobacteria, Firmicutes and bacterioidetes) (Heylen, Vanparys et al. 2006).

A study made by van Rijn (van Rijn, Fonarev et al. 1995) examined the release of volatile fatty acids (VFA, including acetate, propionate and butyrate) from denitrifying bacteria during anaerobic incubation of fresh fish feed in digestion basin medium. It was showed that VFA are major intermediate products during anaerobic degradation of organic matter, and in this experiment the production of VFA was higher than the needed for anaerobic degradation although the experimental reactor was inoculated with fish feed having higher nutrient content than the waste used for aquaculture bioreactors.

1.4.2 Denitrification process

Nitrogen is introduced into the biosphere by biological and chemical fixation of nitrogen (N_2) and removed from there again by denitrification. An N oxide, instead of oxygen, serves as the electron acceptor generating an electrochemical gradient across the cytoplasmic membrane (Zumft, 1997).

Biological nitrate removal is accomplished by two major processes: assimilatory and dissimilatory nitrate reduction.

The dissimilatory branch comprises ammonification in addition to denitrification, being both initiated by respiratory nitrate reduction as a mean of ATP production. Ammonification is the reduction of nitrate to ammonia that does not serve the purpose of N autotrophy (Zumft, 1997), and is conducted by mirco-organisms that reduce nitrate by fermentative pathways. In both processes, C/N ratio determines which one will occur, with high C/N ration favouring the ammonification (Timmons and Ebbeling, 2007).

The assimilatory branch is used by the organisms for biosynthesis of cell components in which nitrate is used as a nitrogen source. It is a process that takes place aerobically or anaerobically by both eukaryotic and prokaryotic organisms (Timmons and Ebeling, 2007).

Table 2, shows the dissimilatory and assimilatory branches of nitrate reduction in the prokaryotic N cycle, with the chemical equations implied in each step.

Table 2: and assimilatory branches of nitrate reduction in the prokaryotic N cycle (Zumft, 1997)

Dissimilatory branch		Assimilatory branch
Denitrification (energy conservation, electron sink)	Ammonification (electron sink, detoxification, energy conservation)	Assimilation (biosynthesis of nitrogen-containing compounds)
Respiratory nitrate reduction $NO_3^- \rightarrow NO_2^-$ (nitrite excreted or reduced further)		Assimilatory nitrate reduction $NO_3^- \rightarrow NO_2^-$ (nitrite reduced further)
↓	↓	↓
Denitrification sensu stricto, nitrite and nitric oxide respiration $NO_2^- \rightarrow NO \rightarrow N_2O$ (gases may be set free)	Ammonifying nitrite reduction $NO_2^- \rightarrow NH_4^+$ (ammonia excreted)	Assimilatory nitrite reduction $NO_2^- \rightarrow NH_4^+$ (ammonia incorporated)
↓	↑	↑
Nitrous oxide respiration $N_2O \rightarrow N_2$	Denitrification sensu lato associated with both branches $NO_3^-/NO_2^- \rightarrow N_2O$	

Among the denitrifiers, the type and quantity of carbon compounds influences the accumulation of intermediate products, when the denitrification is not complete (products like, nitrite, nitric oxide) (van Rijn, Tal et al. 2006). The C/N ratio required for denitrification depends on the nature of the carbon source and the bacterial species that could result in an incomplete denitrification.

Some denitrifying bacteria may use inorganic compounds, such as inorganic sulphur and iron compounds or hydrogen as electron donors, these are the autotrophic denitrifying bacteria (van Rijn, Tal et al. 2006).

The denitrifying process is affected by various factors, such as temperature, pH, salinity, Oxygen, carbon source and C/N ratio. It is also known that denitrification acts on the buffering capacity of the culture water. In recirculating systems, intensive nitrification leads to an alkalinity loss and a resulting pH decline, with the denitrification process some alkalinity is gained (van Rijn, Tal et al. 2006).

1.4.3 Denitrification and Recirculation Aquaculture Systems

As it was said before, RAS concept is based on water re-use. This re-use will result in accumulation of non desirable substances, such as nitrate, the end product of nitrification.

The two most used techniques for nitrate removal are water exchange and/or denitrifying reactor. On the first one, nitrate is removed by changing part of the culture water by new water, increasing the percent of new water used. This method is not considered environmentally friendly, furthermore the effluent discharged have to be under certain limits of nitrate concentrations, settled by the European Council.

On the second one, using a denitrifying reactor can represent advantages in various perspectives. For instance, on an environmental perspective the new water input is lower, as the nitrate concentration of the water discharge.

On a production perspective, denitrification is known to stabilize the buffering capacity of the culture water, compensating the loss in alkalinity during nitrification (van Rijn, J. in Timmons and Ebeling, 2007).

Also, in some RAS area the oxygenation is very low, this can trigger denitrifying bacteria, since the enzyme that reduces nitrate to nitrite is less sensitive to oxygen, than in those micro-sites nitrite production occurs, becoming a problem in the culture water (Arbiv and van Rijn 1995).

It is known that the growth rates of turbot in recirculation systems are about 15 to 20% lower than rates in flow-through systems, this is believed to have a direct relation to the accumulation of substances in the culture water. As it was said before, high nitrate concentrations can affect fish growth and can act has an endocrinologycal disruptor. Applying denitrifaction will remove not only the excess of nitrate, but also the amount of organic carbon and sulphide from the culture water (van Rijn, Tal et al. 2006).

It also known that researchers set hypotheses on the removal of cortisol from the water culture, by the denitrifying bacteria. More recently studies in sewage treatment have been made towards the evaluation of denitrifying bacteria capacity to remove estrogens and other hormones from the water. Talaner and Denner (2003), made an experiment with one bacterial strain (Chol-1S^T) capable of oxidizing cholesterol and reducing nitrate to nitrogenous gas, given the following formula:

$$20C_{27}H_{46}O + 149NO_3^- + 149H^+ \longrightarrow 135C_4H_7O_3 + 74,5N_2 + 62H_2O$$

They found that these bacteria reduced the amount of cholesterol to almost half the concentration in water. They isolated these bacteria from sludge of an USB treating sanitary landfill leachate in Uruguai. This experiment, shows the denitrifying bacteria capacity of removing cholesterol, which origin by steroidogenesis, steroid hormones like cortisol. By removing the precursor of cortisol it is possible that denitrifying bacteria are also able to remove cortisol or other steroids from the water.

1.5. Conclusions

It is widely acknowledged that fish supplies from the world fisheries are unlikely to increase and the expansion of the aquaculture sector will provide the solution for the problem, but with the increasingly limited access to costal areas due to tourism and recreational activities, pressure is put on the aquaculture sector to develop towards a higher sustainability, both economic and environmental.

The utilization of RAS is a higher priority in the development for aquaculture sector, this development is attached with a higher environmental sustainability, reducing the amount of water discharged. The low level of new water coming into the system, allows the accumulation of non-desirable substances, such as nitrate (the end product of nitrification), this seems to be the reason why growth rates of turbot are lower in RAS than in flow-through systems.

Few literature is available on the effect of nitrate in growth. As referred before, nitrate seems to affect growth and can act as an endocrinological disruptor. Due to this nitrate should be removed from the culture water. Besides the capability of denitrifying bacteria to reduce nitrate to nitrogen, they also produce free amino acids and studies are being made in order to evaluate the capacity of cortisol removal from the water column, therefore removing GIF from the culture water. It is also believed that the usage of a denitrifying reactor could produced GIF neutralizing substances, such as humic acids that could attach to steroids or bind with possible environmental stressor like toxic metals, making them unsoluble in water, therefore undetected by fish.

2. Objectives and hypothesis

RAS is raising its importance on the aquaculture sector, due to a more environmentally friendly concept and trying to achieve a more economical sustainability. One of the major targets to this attainment is to overtake the growth retardation that seems to happen in RAS.

The main goal of this experiment was to evaluate the growth performance of turbot reared under the same concentration of nitrate, but one treatment took place with a USB reactor and a low water exchange rate (theoretically 30l/kg of feed) and the other treatment (control treatment) occurred without a USB reactor and a higher water exchange rate (300 L/kg of feed).

This experiment is also able to supply information for USB reactor performance, with the capacity to be used on other fish culture reducing the wastewater.

Hypothesis

H_o.: There is no significant difference on growth performance of turbot between treatments.

H_a.: There is significant difference on growth performance of turbot between treatments.

Towards the sustainable development of aquaculture sector, reducing waste in water discharge and reducing the water discharges it self is one important step. This experiment focuses also in the integration of a USB reactor as a mean to diminish the waste per kg of feed and also reducing the water exchange in a sustainable production system. For this, in the experiment a USB reactor was used accomplished with a low water exchange, maintaining the nitrate concentration at 150 mg/l.

H_o.: USB reactor is not able to maintain the nitrate concentration at 150 mg/l, with a water exchange of 30 L/kg of feed.

H_a.: USB reactor is able to maintain the nitrate concentration at 150 mg/l, with a water exchange of 30 L/kg of feed.

3. Material and Methods

3.1 General description of the experimental design

The elaboration of this experiment had the purpose to study the influence of an USB reactor on the growth performance of turbot, cultured in recirculation systems and the efficiency of it with low water exchange rate (set to be 30 L/Kg of feed).

This experiment was designed to start on the 21st of August and end on the 16th of October, having a total duration of 8 weeks. An adaptation period of two weeks was made previously. The experiment consists in 6 small scale recirculation aquaculture systems, stocked with 22 randomly chosen turbot each, making a total of 132 fish, during the adaptation period. At the beginning of the experiment, fish were weighted and two fish per system were removed for body composition analyses. At the end of the experiment fish were also weight, after a fasting of 24 hours for calculation of growth performance and sampled for body composition analyses.

The experimental design consists in two treatments in triplicate, one treatment with a higher water exchange rate of 300 L/Kg of feed and the other treatment with a lower exchange rate and an USB reactor. Table 3 represents the parameters for both treatments, describing the variation between treatments.

Table 3. Parameters for control and RAS USB treatment

	Control treatment	**RAS USB treatment**
Number of fish (per tank)	20	20
Initial average weight (g)	448,6±11,83	420,8±20,22
Replicates	3	3
Duration (days)	54	54
Nitrate concentration (mg/l)	150	150
Water exchange (l/kg feed)	300	to be determined

3.2 Fish

Fish was originated from the company France Turbot (date of hatching 16/06/2007) and transferred to a commercial farm in the Netherlands (GroVisCo, Stavenisse, The Netherlands). Before fish were transported to the experiment facility (Haar Vissen, Wageningen University, Netherlands) they were kept in small aquaria for 10 weeks and then transferred to tanks of 1.1 m^3 during other ten weeks, at the Haar Vissen hatchery of the Wageningen University. Previously to the start of the experiment, two weeks of adaptation period occurred.

Fish were stocked, at the first day of the experiment with 425 DPH and an average weight of 435g.

3.3 Feed

The feed used in this experiment was a commercial feed from the company DANA FEED A/S. now belonging to BioMae A/S. The feed content of protein, fat, carbohydrates and phosphorus declared by the manufacturer is, respectively, 54%, 18%, 10.5%, 1.46%. The complete composition of the feed is presented in table 4. The feed given to the experimental fish were extruded pellets with 9 mm, and an individual average weight of 0.5g.

Table 4. Table with the ingredients percentage in feed used in the experiment

DAN-EX 1562 9.0 mm, DANA FEED A/S	
Content	
Crude Protein	54 %
Crude Oils and Fat	18 %
Carbohydrates	10.5%
Crude ash	9.9%
Fibre	0.9 %
Phosphorus	1.5 %
Additives	
Vitamine A	1.01 I.U/g
Vitamine D	0.16 I.U/g
Vitamine E	277 mg/kg
Cu	7.6 mg/kg

Raw materials	
Fish meal	66.72 %
Wheat	11 %
Fish oil	10.65 %
Soya bean	6 %
Soya bean protein concentrate	5 %
Vitamin premix	0.63 %

3.4 Feeding strategy

The fish were fed twice a day by hand until near satiation. The first feeding was in the morning (9 am) and the second in the afternoon (5 pm), seven days a week. By feeding the fish by hand, there is a better perception of the overall feeding, reducing waste caused by non eaten pellets. To get the amount of eaten feed, every day the feed distributed by the fish tanks were weight previously and after feeding. After each feeding period, the remaining pellets were counted and weight estimated. By the feeding motivation, inspected visually, fish health was taken into account.

3.5 System design and operation

The experiment operated in recirculation systems, composed by a fish tank with a central outlet. The water from the fish tank flows to a Hydro-cyclone tank of 100 L that works as a sedimentation unit, following to a sump with U.V. filtration, and then the water is pumped to the trickling filter, descending by gravity into the fish tanks.

Six small scale recirculation systems were used; three of them with a USB reactor with a low water exchange rate (theoretically 30L/Kg of feed); the control treatment operated without the USB reactor and with a normal flow rate of 300L/Kg of feed.

The table (table 5) below represents the characteristics of the RAS components, and the following figures display an experimental schematization and disposition of RAS units on the treatments, USB reactor and control, figure 1 and 2 respectively. Figure 3 represent a closer look to the USB reactor, with the inlet and outlets marked with letters.

Evaluation of the performance of turbot (*Psetta maxima* L.) in RAS with and without an Upflow Sludge Blanket reactor.

Table 5: Resume of RAS components describing characteristics, function and observations.

RAS component	Volume	Function	Observations
Fish tank	Total vol.: 425l Effective vol.: 300l	Tank where fish were kept	Circular tank, with central stand pipe (outlet)
Sedimentation tank **Hydro-cyclone**	Volume: 100l	Collect the accumulation of solids in the culture water, by the of centrifugal force and gravity	• TSS> 77 µm: 87% efficiency (Scott and Allard, 1984). • Treatment with USB, had a stirrer that automatically (every half hour) removed the content of the hydro-cyclone. • A flask under the hydro-cyclone were used to collect faeces for digestibility, in the control treatment; the treatment with USB the flask were connected to centrifugal pump that pumped to the inlet of the USB
Sump with U.V. **UV-C: 36 watts philips**	Volume: 100l	Disinfection by UV radiation.	• UV light acts damaging directly or indirectly nucleic acids (Timmons and Ebeling, 2002) • The sump was equipped with a floater to control water level.
Pump		Transport water from the UV sump to the cooler/heater	
Cooler/heater TC20, TECO®		Maintain water temperature	
Light	36watts/840	Mimic day and night	• Photoperiod was set at 18:6 (light:dark)
Trickling filter	Volume: 0,054 m^3 Area: 10,8m^2 Dimensions: 35x35x70 cm	Biofiltration, presence of nitrifying bacteria (nitrification)	• Filled with plastic media (Bio-net, Catvis BV) already inoculated with bacteria coming from other experiment with turbot (DEC 2007061) • Water coming from the cooler/heater, descended by gravity to a plate were the water was equally distributed on the top of the trickling filter.
USB reactor	Volume: 10,5 l Cylinder: 1,98 m high; 9 cm ∅ Upflow rate: 0,6m/h Flow water inlet: 3,6 l/h	Biofiltration, presence of denitrifying bacteria (denitrification)	• Solids collected into the flasks under the hydro-cyclone were pumped to the bottom of the USB (0,04m above the bottom) • The top of the USB is connected to the hydro-cyclone, making a recirculation between both • A stirrer with 1,74 m, stirrer the sludge every 30 secs during 30 secs, permitting gas removal and mixing the sludge. • The inoculation of the reactor was maid at the first day of experiment, with no adaptation period. Sludge came from other RAS with turbot (DEC 2007061)

Evaluation of the performance of turbot (*Psetta maxima* L.) in RAS with and without an Upflow Sludge Blanket reactor.
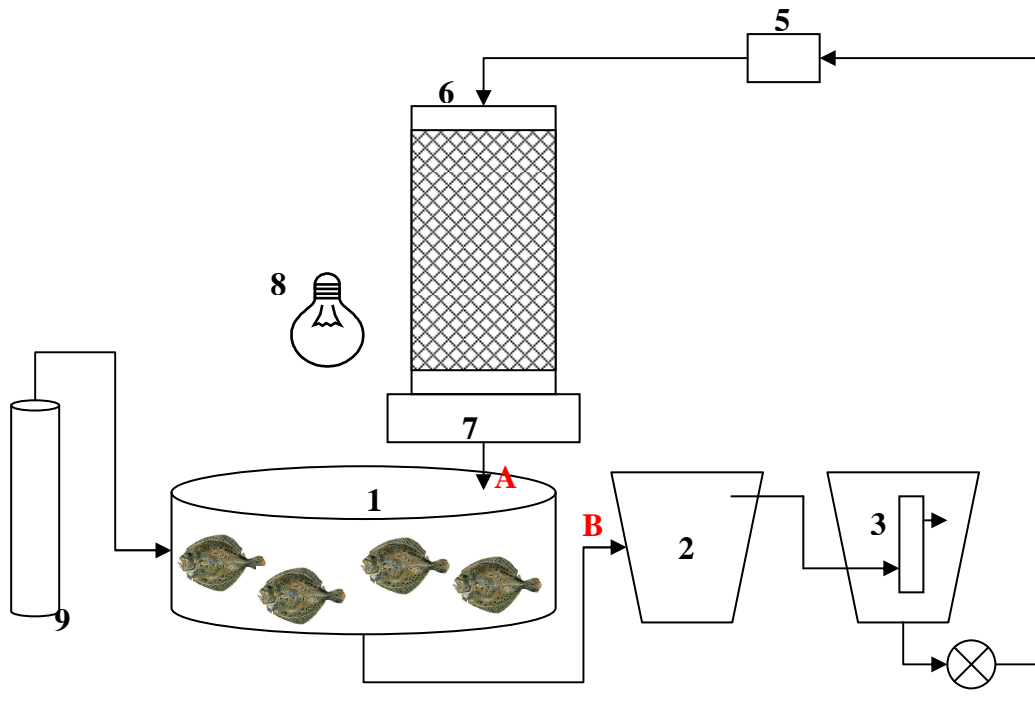
Figure 2. Experimental schematization of the system with USB reactor (Jauffrais, 2008), 1: circular fish tank (300 L), 2: sedimentation unit of 100 L (Hydro-cyclone), 3: sump with UV of 100 L, 4: pump, 5: cooler-heater, 6: trickling filter (54 L), 7: sump, 8: light, 9: emergency O2; A and B sampling places
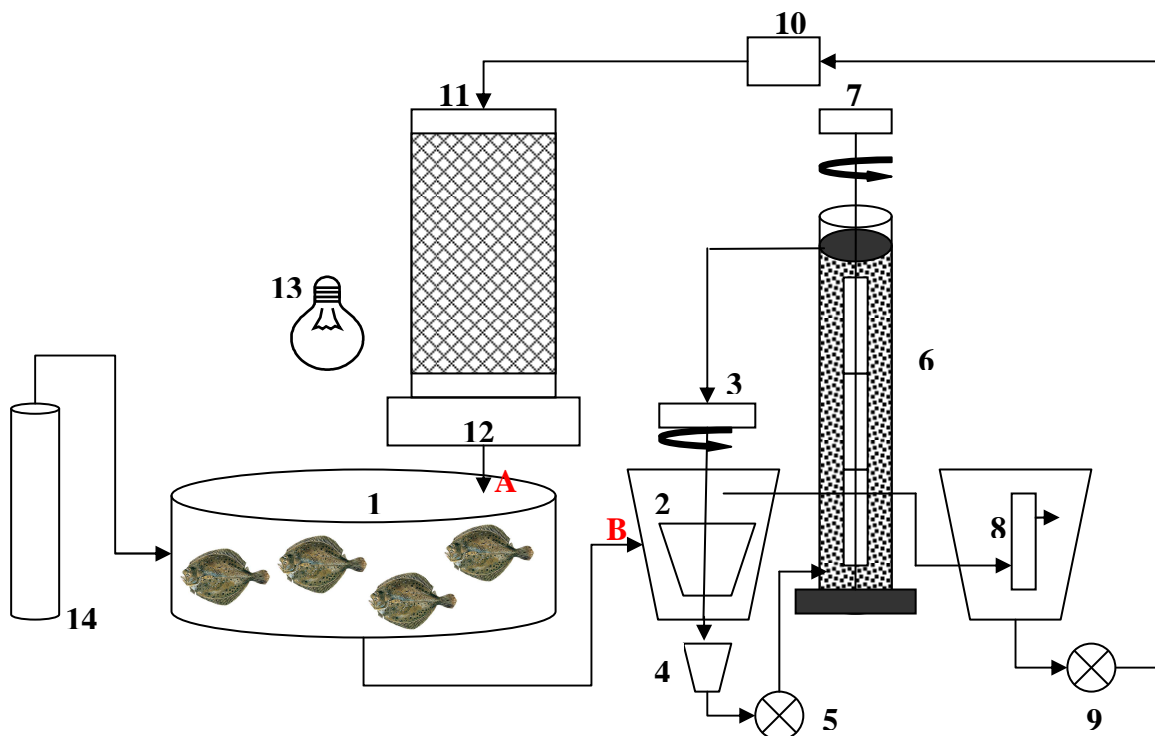
Figure 3. Experimental schematization of the system with USB reactor (Jauffrais, 2008), 1: circular fish tank (300 L), 2: sedimentation unit of 100 L (Hydro-cyclone), 3: stirrer, 4: stirrer, 5: pump, 6: USB (10.5 L), 7: stirrer, 8: sump with UV of 100 L, 9: pump, 10: cooler-heater, 11: trickling filter (54 L), 12: sump, 13: light, 14: emergency O_2; and A and B sampling places
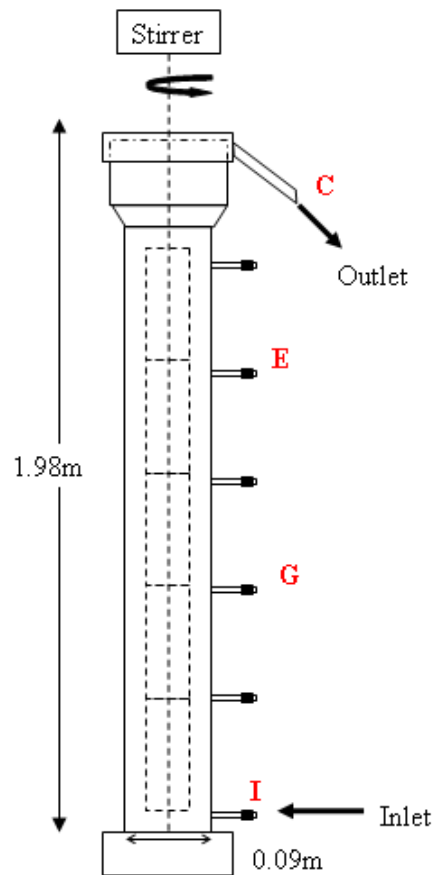
Figure 4. Experimental schematization of the Upflow Sludge Blanket (Jauffrais, 2008), with the collecting points represented by the letters

3.6 Measurment and analyses

3.6.1 Water Quality

To ensure the same water quality in both treatments, several water parameters were measured at daily or weekly. The measurements took place in the morning before first feeding.

To maintain the value nitrate concentration in all systems, a water discharge was applied after every feeding. In the control systems the exchange rate was 300 l/kg of feed, in the USB systems the exchange rate was 30 l/kg of feed. But since the nitrate concentration should be maintained at 150 mg/l, these water exchanges could vary exchanging higher quantities when nitrate concentration exceeded the limit.

During the 24 hour measurement, on a weekly basis, a 150 ml sample was collected every four hours for measurements on SAN autoanalyser (SKALAR). All samples were kept at 4º C until analysis within 24 hours.

The table 6, represent the measurement made for water quality with daily and weekly (during 24 hours), the instrument used, the place where it was measured and the optimum or limit value for turbot growth.

Table 6: Measurements for water quality, showing the frequency, instrument used the place of measurement in the system and the optimum or limit value for the correspondent parameter.

Measurement	Frequency	Instrument to measure	Place of measurement	Optimum/ limit value
Temperature	Daily	WTW, cond 340i	Fish tank outlet; USB outlet	18º C
pH	Daily	WTW pH 340	Fish tank outlet; USB outlet	7 – 7,5
Dissolved Oxygen	Daily	WTW, pH/OXI 340i	Fish tank	>6 mg/l
Salinity	Daily	WTW, cond 340i	Fish tank outlet	15 ‰
TAN	Daily	Merck test	Fish tank outlet; USB outlet	< 8,8 mg/l
	Weekly	SAN autoanalyser	Fish tank outlet; USB outlet/inlet	
Nitrite-N	Daily	Merck test	Fish tank outlet; USB outlet	< 0,5 mg/l
	Weekly	SAN autoanalyser	Fish tank outlet; USB outlet/inlet	
Nitrate-N	Daily	Merck test	Fish tank outlet; USB outlet	150 mg/l
	Weekly	SAN autoanalyser	Fish tank outlet; USB outlet/inlet	
Urea	Weekly	SAN autoanalyser	Fish tank inlet/outlet	**-**
Bicarbonate	Weekly	SAN autoanalyser	Fish tank inlet/outlet	**-**
Ortho-phosphate	Weekly	SAN autoanalyser	Fish tank inlet/outlet	**-**

3.6.2 USB Reactor

Temperature, pH, TAN, nitrite and nitrate, were measured daily in the USB, to monitor the performance (Table 6). Water flow was also measured daily, twice during one minute. The level of sludge in the USB was also monitored every day.

To check the performance of the USB, samples were collected weekly during 24 hours, collecting 6 times 10.8 L first in the outlet and then in the inlet (Figure 4) during 30 minutes each sample and stored in ice for preservation. After that and while stirring the sample a sub sample of 300 L were collected and kept at 4ºC for further analysis.

In the next morning, 50mL of sludge were collected at the different sampling outlet of the USB (Figure 4, collected in port E, G and I by this order), then 10 ml were collect in the same procedure for water analysis.

3.6.3 Analytical technique

The samples from the USB were divided in two. The sludge samples collected at outlet, inlet and various portics were used to measure dry matter, ash, total suspended solids, volatile suspended solids, Kjeldahl-nitrogen, ortho-phophate and total carbonate. The water samples collected at various portics were used for measurements of, TAN, nitrite, nitrate, total carbonate, ortho-phosphate and urea.

All the measurement was made according to the protocols of the Aquaculture and Fisheries Group of the Wageningen University.

3.7 Fish performance

The performance parameters were measured based on the experiment period of 54 days. At the first day the fish were weighted and placed randomly in each tank. At the final day of the experiment fish were anesthetized and weighted again. The following parameters were calculated: total initial and final biomass; survival; growth (**G**); specific growth rate (**SGR**); metabolic body weight (**MBW**); relative growth rate per metabolic body weight (**RGRm**); feed conversion ratio (**FCR**); feed intake (**FI**); relative feed intake (**RFI**);

metabolic feed intake (**MFI**); relative feed ratio per metabolic body weight (**RFRm**), described in table 7. Fish composition and digestibility were also analyzed.

Table 7: Parameters calculated and their formula

Parameter	Formula	Abbreviations
Survival	$(N_{final}/N_{initial})*100$	N_{final} is the final number of fish at day 54; $N_{initial}$ is the number of fish at day 0
G	$(W_{final} - W_{initial})/(tf-ti)$	G: growth; W_{final} and $W_{initial}$ is the weight of fish at day 54 (tf) and day 0 (ti), respectively
SGR (%)	$(lnW_{final} - lnW_{initial})/ (tf-ti)*100$	SGR: specific growth rate
MBW (kg^{0,8})	$(e^{((lnWinitial+ lnWfinal)/2)})/1000^{0,8}$	MBW: metabolic body weight
RGRm (g/ kg^{0,8}/day)	G/MBW	RGRm: relative growth rate per metabolic body weight
FI g/fish/day	$(Fm+Fa-Fw)/(20*(survival/100))$	FI: feed intake; Fm, Fa, Fw amount of feed in the morning, afternoon and wasted respectively
RFI	$FI/(W_{fina}– W_{initial})^{0,5}/(tf-ti)$	RFI: relative feed intake
RFR (g/ kg^{0,8}/day)	FI / MBW/(tf-ti)	RFRm: relative feed ratio per metabolic body weight
FCR (g feed/g fish)	RFR/RGRm	FCR: feed conversion ratio

3.8 Digestibility

To calculate digestibility it was analyzed the feed composition and the faeces composition.

3.8.1 Feed composition

Samples of the commercial feed used during the experiment were mixed and grinded in a mill with a sieve screen mesh sized 1 mm, enabling the chemical analysis for

measurements of dry matter, ash, crude protein (Kjeldahl-nitrogen method, total nitrogen was calculated as N=protein/6,25), acid insoluble ash (AIA), gross energy, fat and total phosphate.

3.8.2 Faeces composition

To analyze the faeces composition, they were collect during a period of 48 hours starting at the same time of the 24 hour measurement. The faeces were collected to a flask attached under the hydro-cyclone on the control systems, this flasks were kept on ice during the collection, to avoid nutrient loss by bacteria degradation. Then the faeces were collected to aluminum boxes after each feeding (morning and afternoon) and stored in the freezer at -20º C, until further analysis. For analysis, faeces were freeze dried and grinded in a mill with a mesh screen size of 1 mm enabling chemical analysis. Faeces were measured for dry matter, ash, crude protein (Kjeldahl-nitrogen method), acid insoluble ash (AIA), gross energy, chemical oxygen demand (COD) and total phosphate.

To calculate efficiency of faeces/feed waste on the sedimentation tank (hydro-cyclone), efficiency of AIA recovery were calculated using the following formula:

AIA efficiency of recovery $= (AIA_{feed} - AIA_{faeces}) / AIA_{feed}$

Where: AIA_{feed} and AIA_{faeces} are Acid Insoluble Ash in the feed and faeces, respectively

3.8.3 Apparent digestibility

The method used to measure the digestibility in this experiment is an indirect method based on using an inert marker (AIA) comparing the feed composition with faeces composition. The apparent digestibility was calculated by the formula:

ADC $= 100-(100*(AIA_{feed}/AIA_{faeces})*([N]_{faeces}/[N]_{feed}))$

Where: AIA_{feed} and AIA_{faeces} are Acid Insoluble Ash in the feed and faeces, respectively, and $[N]_{faeces}$ and $[N]_{feed}$ represent the amount of nutrient in analysis in the faeces an feed, respectively (Jobling, M. 1994).

3.8.4 Real digestibility

In this calculation, the amount of feed waste is taken into account. Feed waste was calculated as the sum of each feeding waste, from 12 hours before the collection started until 12 hour before the collection ended. On the third week, the collection of faeces was during plus 12 hours due to the little amount of faeces collected. The formula used for real digestibility calculation was:

$$RD = 100\text{-}(100*((100*[N]_{feed}/1000)*(100\text{-} ADC_N/100)\text{-}([N]_{feed}/1000*Wst))/(100\text{-}Wst)*([N]_{feed}/1000)$$

Where $[N]_{feed}$ represents the amount of nutrient in analysis in the feed, ADC_N represents the apparent digestibility coefficient of the nutrient, Wst represents the percentage of waste as feed spillage (Heinsbroek, L.).

3.8.5 Fish body composition

The fish body composition was determined at the beginning and at the end of the experiment. At the beginning, two fish per tank were sacrificed and frozen after being frozen, they were cut in peaces, grinded and mixed for chemical analysis. At the end all fish were sacrificed, and five fish were applied the same procedure as in the beginning of the experiment.

The fish were used for measurements of dry matter, ash, crude protein, energy, fat and total phosphate. The nutrient retention was calculated with the following formula:

$$Ret_N = W_f*(N_f/1000)\text{-}W_i*(N_i/1000)$$

Where W_f and W_i correspond to the individual weight at the final and at the beginning of the experiment, respectively. N_f and N_i correspond to the amount of nutrient to calculate, in the body composition of the fish (g of nutrient/kg of fish).

3.9 Energy budget

Energy budget was calculated for each system, in order to determine the energy requirement for maintenance. All energy values are present as $KJ/kg^{0.8}/day$

Gross energy intake GE $= FI/[GE]_{feed}/(tf-ti)$

Where $[GE]_{feed}$ is the concentration of energy in feed KJ/g; FI is the feed intake; tf and ti is final and initial days of the experiment.

Digestible energy DE $= GE \times ADC_E/100$

Where ADC_E is the apparent digestibility coefficient of Energy (%)

Fecal energy loss FE $= GE - DE$

Branchial Urinary Energy BUE $= BUN \times 24,85$

Where BUN is branchial urinary nitrogen = digestible nitrogen – retained nitrogen; 24,85 KJ.g/N (Heinsbroek, 1989)

Metabolize energy ME $= DE - BUE$

Retained energy $= W_f*E_f-W_i*E_i$

Where, W_f and W_i correspond to the individual weight at the final and the beginning of the experiment, respectively. E_f and E_i correspond to the amount of energy in fish body composition fish (KJ).

Total heat production H $= ME - RE$

Energy requirement for maintenance MEm $= H - [RE*(1-k_g)]$

Where k_g, is the efficiency of energy retention by fish.

3.9 Mass balances

Inorganic nitrogen (N) and phosphorus (P) are immobilized in bacterial biomass or volatized and/or discharged in a less hazardous form in RAS. The majority of the organic and inorganic waste is discharged as sludge through the effluent. These nutrients are used by the denitrifying bacteria (Schneider, Sereti et al. 2007). To understand the performance of the RAS system and USB reactor, the nitrogen phosphorous and chemical oxygen demand was calculated by the following equations.

3.9.1 Nitrogen mass balance

The formula used for calculating nitrogen mass balances is presented below:

$$N_{feed} = N_{growth} + N_{removal\ USB} + N_{discharged\ solid} + N_{accumulated\ USB} + N_{accumulated\ system} + N_{unexplained}$$

N_{feed} : Nitrogen supplied in the feed to the fish

N_{growth} : Nitrogen retained in the fish

$N_{removal\ USB}$: Nitrogen removed by the USB

$N_{discharged\ solid}$: Nitrogen in the feed waste and faeces, corrected for efficiency of sedimentation

$N_{accumulated\ USB}$: Difference of total nitrogen in the USB at the first and last day

$N_{accumulated\ system}$: Difference of total nitrogen in the system at the first and last day

$N_{unexplained}$: Nitrogen removed that can not be explained by the model

3.9.2 Phosphate mass balance

The formula used for calculating phosphate mass balances is presented below:

$$P_{feed} = P_{growth} + P_{discharged\ solid} + P_{water\ refreshment} + P_{accumulated\ USB} + P_{accumulated\ system} + P_{unexplained}$$

P_{feed}: Phosphate supplied in the feed to the fish

P_{growth}: Phosphate retained in the fish

$P_{discharged\ solid}$: Phosphate in the feed waste/faeces, corrected for efficiency of sedimentation

$P_{water\ refreshment}$: Phosphate in the water discharge

$P_{accumulated\ USB}$: Difference of total phosphate in the USB at the first and last day

$P_{accumulated\ system}$: Difference of total phosphate in the system at the first and last day

$P_{unexplained}$: Phosphate that can not be explained by the model

3.9.3 Chemical Oxygen Demand mass balance

Due to most part of the bacterial biomass is organic material, the increase in biomass can be measured by volatile suspended solids or particulate COD (COD_{total}-$COD_{soluble}$). The

COD of cell tissue is assumed to be, 1,42 g O_2/g of cells (Tchobanoglus, G., Burton, F. et al, 2002). The formula used for calculating COD mass balances is presented below:

$$COD_{feed} = COD_{growth} + COD_{discharged\ solid} + COD_{respirationo\ in\ fish} + COD_{respiration\ in\ USB} +$$
$$COD_{accumulated\ USB} + COD_{unexplained}$$

COD_{feed}: COD supplied in the feed to the fish

COD_{growth}: COD retained in the fish

$COD_{discharged\ solid}$: COD in the feed waste/faeces, corrected for efficiency of sedimentation

$COD_{respiration\ in\ fish}$: oxygen consumed by fish for maintenance and growth

$COD_{respiration\ in\ USB}$: COD of organic matter removed in denitrification

$COD_{accumulated\ USB}$: Difference of COD in the USB at the first and last day

$COD_{unexplained}$: COD that cannot be explained…

N.B. in COD unexplained is also all dissolved COD which was not measured.

3.10 Statistical analysis

Statistical analyses were conducted using SPSS. All results are expressed as mean ± standard deviations. Significant differences within a group were analyzed with student t test when data are normally distributed and with Mann-Whitney test when data are not normally distributed.

For weekly water quality and feed intake one-way ANOVA for repeated measurements with system age as main factor and time as sub-factor.

Analysis of covariance was realized to determine the differences between curves for water exchange, nitrogen and phosphate production, and sodium bicarbonate consumption, the following model was used: $y = \mu + Age + \beta_1*Feed + \beta_2*(Age*Feed) + e$

Differences are considered significant when $p<0,05$ and highly significant at $p<0,01$.

4 Results

4.1 Fish growth performance

Fish performance was evaluated based on several parameters, showed in table 8, values are presented in mean±standard deviation. Regarding to growth there was no significant difference between treatments in the initial and in the final individual weight, the same was observed with metabolic body weight. Significant differences were observed in specific growth rate (%BW/day), relative growth rate by metabolic body weight (g/kg^{0,8}/day). These parameters were higher in RAS USB treatment.

Regarding to feed intake there was no significant difference between treatments, also relative feed intake by metabolic body weight showed no significant difference. Feed conversion ratio showed significant difference between treatments being lower in RAS USB treatment.

Table 8: Values for initial and final weight, total biomass; survival; growth; specific growth rate (**SGR**); relative growth rate of metabolic weight (**RGRm**); Absolute feed ratio; Feed intake (**FI**); relative feeding of metabolic weight (**RFR**); Feed conversion ratio (**FCR**); conversion efficiency. P<0,05 indicates significant differences between treatments (T-student test).

	Treatment (Mean±standard deviation)		
Growth	**Control**	**USB**	**P-value**
Initial ind. weight (g)	448,6±11,83	420,8±20,22	0,11
Final ind. weight (g)	575,9±5,71	592,7±19,43	0,22
Total biomass produced (kg)	2,35±0,49	3,04±0,28	0,12
Survival (%)	99,0±1,68	98,3±2,13	-
Absolute growth (g/day)	2,4±0,15	3,2±0,12	0,11
SGR (%BW/day)	0,46±0,04	0,63±0,04	**0,01**
RGRm (g/kg^{0,8}/day)	4,1±0,31	5,6±0,30	**0,00**

Feed intake			
Absolute (g/day)	42,4±4,75	42,2±3,17	0,98
Feed intake (g/fish/day)	2,2±0,21	2,2±0,21	0,99
RFR (g/kg^{0,8}/day)	3,7±0,41	3,8±0,39	0,86
FCR g /g	0,92±0,08	0,68±0,04	**0,00**
Conversion efficiency (%)	109,2±9,13	140,2±19,87	**0,00**
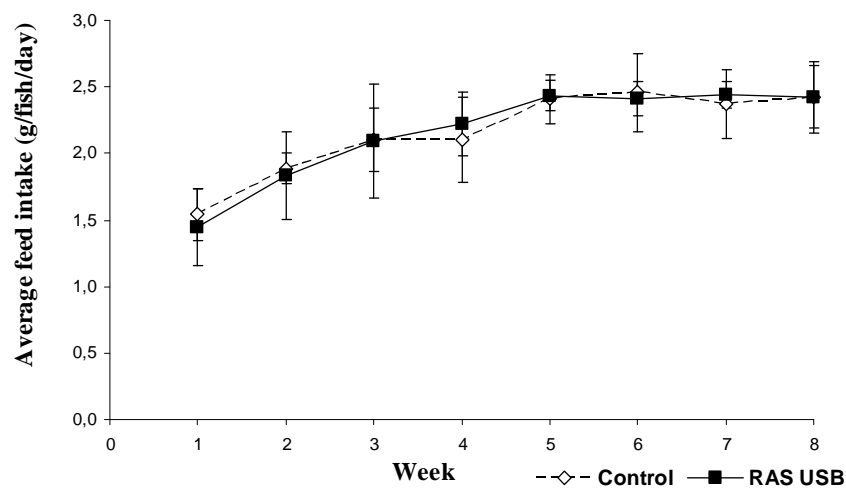
Figure 5: Feed intake of turbot for control and RAS with USB systems.

Figure 5 shows no significant difference between treatments, regarding feed intake, increasing from the week 1 to week 5 and stabilizing until week 8.

4.2 Water quality

The following table (table 9) presents the value for water quality registered every day of the experiment,

Table 9: Values for temperature, pH, oxygen, salinity for both treatments. P<0,05 indicates significant differences between treatments (T-student test).

Water quality	Treatment (Mean±standard deviation)		P-value
	Control	USB	
Temperature (ºC)	17,3±0,12	17,3±0,12	0.31
pH	7,3±0,11	7,2±0,04	0,00
Oxygen (mg/l)	7,4±0,37	7,3±0,37	0.29
Salinity (‰)	15,3±0,16	15,4±0,21	0,00

4.2.1 Ammonia-N

Figure 6: Weekly mean Ammonium-N concentration (mg/L) in both treatments, (* indicates significant difference between treatments ($P<0.05$), the test was a one-way analysis of variance for repeated measures).

Figure 6, presents the development of ammonia-N concentration along the experimental period, measured weekly in both treatments. Ammonia-N concentration was high for RAS USB treatment, at the beginning of the experiment but with high standard deviations. After the third week, ammonia concentration stabilized until the end of the experiment. Significant differences were observed in week 4, 5, 6 and 8.

4.2.2 Nitrite-N
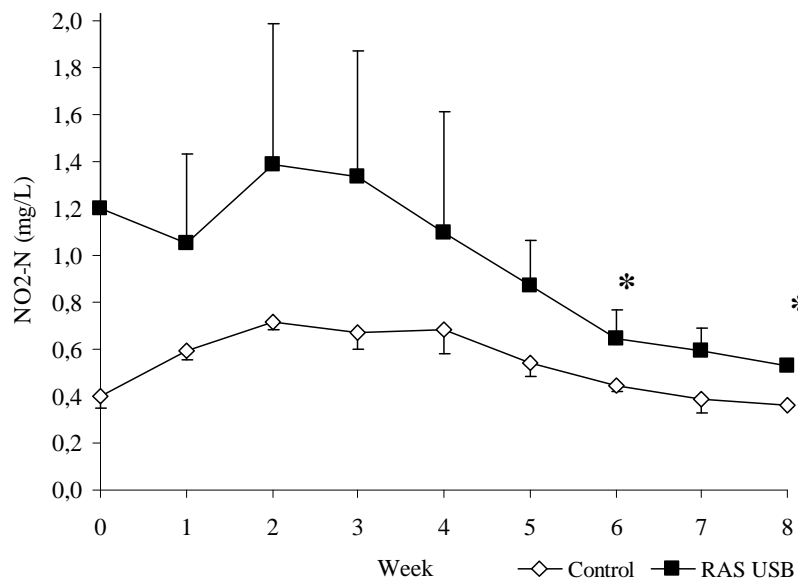
Figure 7: Weekly mean Nitrite-N concentration (mg/L) in both treatments, (* indicates significant difference between treatments (P<0.05), the test was a one-way analysis of variance for repeated measures).

Figure 7, represents the weekly measurement of nitrite-N for both treatments. High standard deviations were observed in RAS USB treatment, mainly due to system 5. The concentration of nitrite-N increased until week 2, decreasing until week 5. After that it stabilized. Nitrate-N concentration was on average higher on RAS USB treatment, with significant differences on week 6 and 8.

4.2.3 Nitrate-N
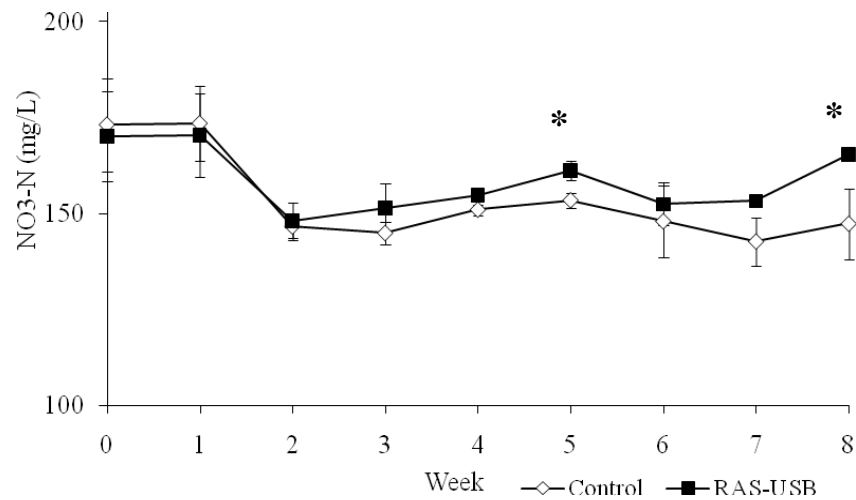
Figure 8: Weekly mean Nitrate-N concentration (mg/L) in both treatments, (* indicates significant difference between treatments ($P<0.05$), the test was a one-way analysis of variance for repeated measures).

Concentration of Nitrate-N was measured weekly, it can be observed on figure 8 that Nitrate-N concentration started above 150 mg/L, as it was referred before, for this experiment Nitrate-N concentration was to be maintained at 150 mg/L due to this an extra water exchange on second week was made to keep the desired concentration. After that the concentration maintained stable until week 8, being slightly higher for RAS USB treatment. Significant difference was observed on week 5 and 8.
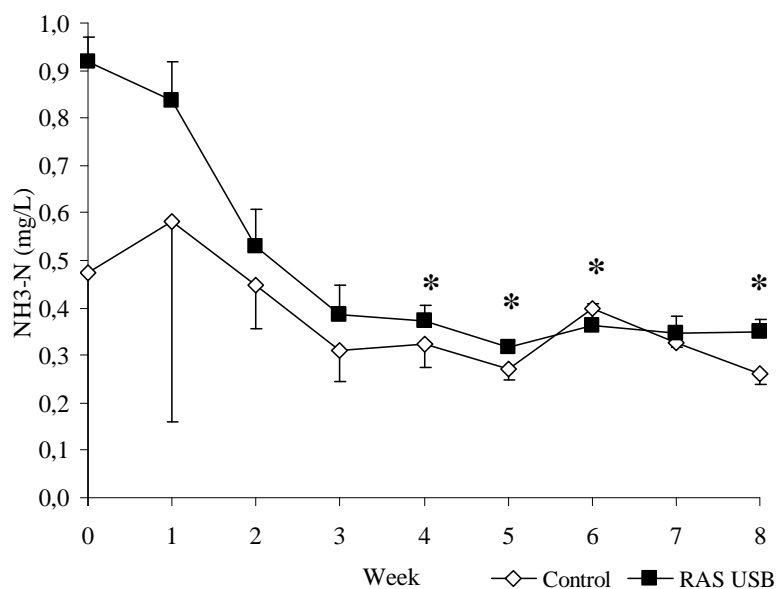
4.2.4 Orthophosphate-P
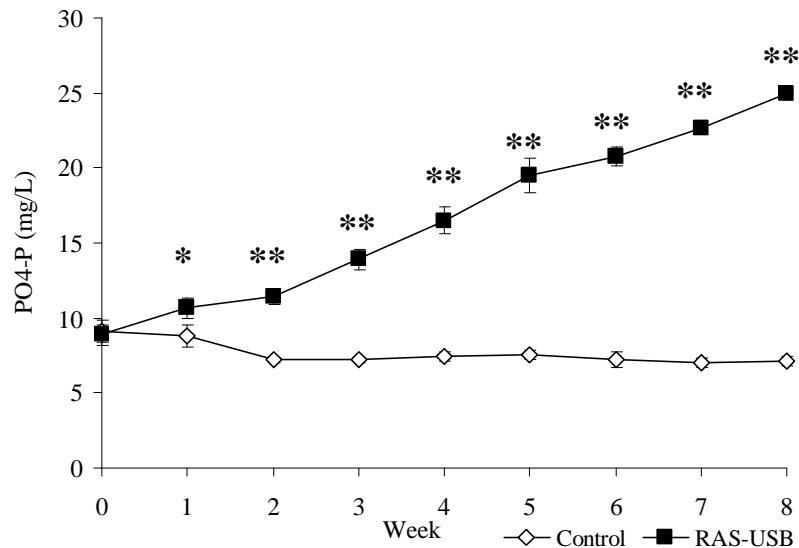
Figure 9: Weekly mean orthophosphate-P concentration (mg/L) in both treatments, (* indicates significant difference between treatments ($p<0.05$), ** indicate highly significant difference between treatments ($p<0,01$) the test was a one-way analysis of variance for repeated measures).

Figure 9 represents the mean concentration of Orthophosphate-P for both treatments. It can be observed that in control treatment the concentration maintained stable during the experimental period. On the other hand, on RAS USB treatment the concentration increased almost constantly during the experimental period with no sign of stabilizing, showing significant differences in all measurements from week 1 until week 8.

4.3 System efficiency

The system efficiency was evaluated with the cumulative water exchange, the cumulative nitrogen production, cumulative orthophosphate-P production and cumulative sodium bicarbonate consumption. All these values were plotted against the cumulative feed given during the experimental period.

4.3.1 Water exchange
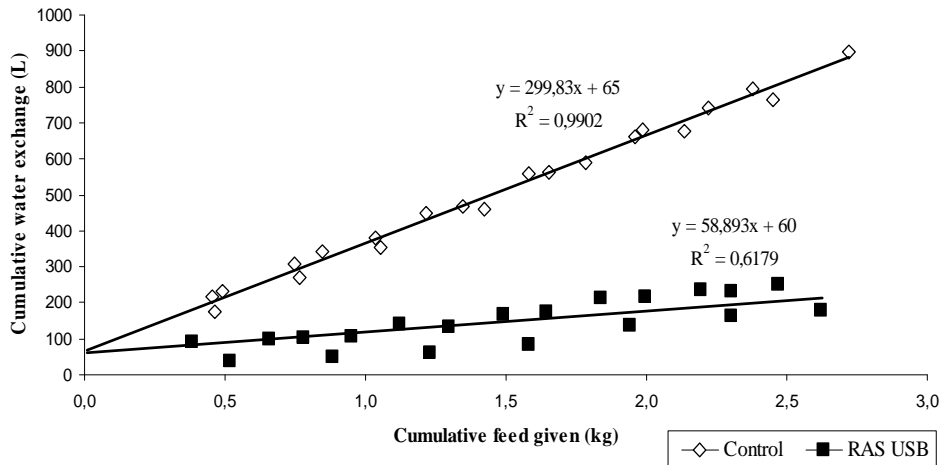
Figure 10: Cumulative water exchange per kilogram of cumulative total feed given for the two treatments (p= 0.00, from the ANCOVA analysis)

Figure 10 presents the cumulative water exchange for all six systems, versus the cumulative feed given. The water exchange for this experiment was set to maintain the nitrate concentration at 150 mg/L, being 300 L/kg of feed in systems without USB and 30 L/kg of feed in systems with USB. Due to the extra water exchange on week one, values from that week were considered outliers. Figure 10, shows that, for the control treatment, the water exchange achieved the expected, almost 300 L/kg of feed (y=299,8x + 65, R^2=0,99). For the RAS USB treatment the water exchange was almost the double of the expected, 58,9 L/kg of feed (y=58,9x + 60, R^2=0,62). Statistical analysis showed significant differences between treatments.

4.3.2 Nitrogen production
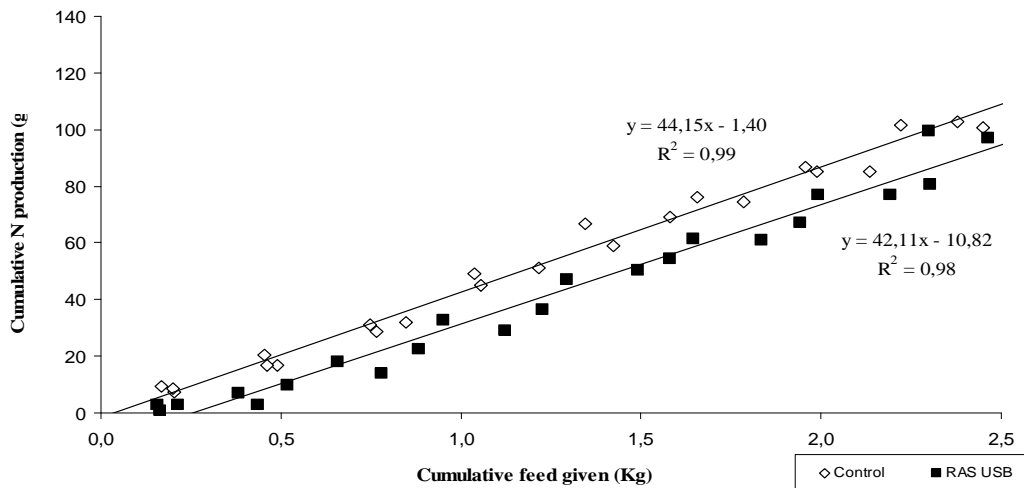
Figure 11: Cumulative nitrogen production (g) per kilogram of total cumulative feed given for the two treatments (p= 0.19, from the ANCOVA analysis)

The cumulative nitrogen production for all systems is presented on figure 11 with the cumulative feed given. It can be observed that nitrogen production is higher in control treatment (44,0 g of nitrogen/kg of feed) than in RAS USB treatment (34,4 g of nitrogen/kg of feed). Statistical analysis showed no significant differences between treatments.

4.3.2 Dissolved phosphate production
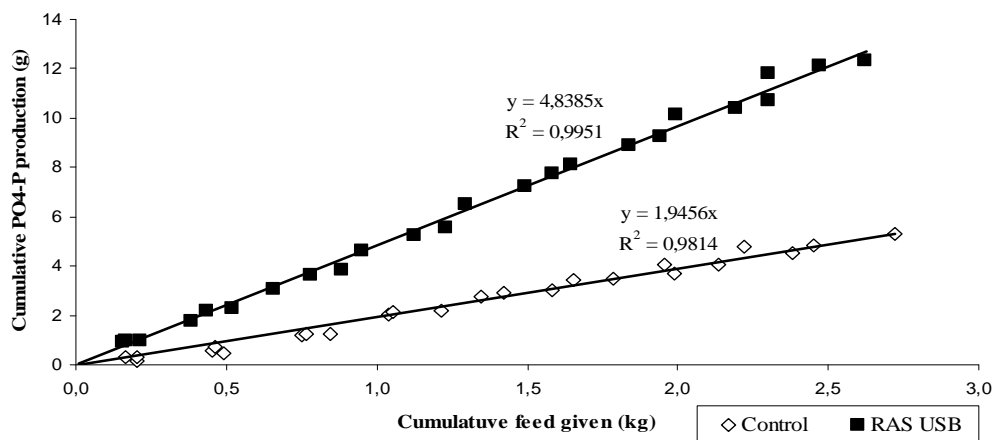
Figure 12: Cumulative orthophosphate-P production (g) per kilogram of total cumulative feed given for the two treatments (p= 0.00, from the ANCOVA analysis)

The cumulative orthophophate-P is presented in figure 12 against the cumulative feed given. For the control treatments the orthophosphate-P production was higher (4,84 g/kg of feed) compared with RAS USB treatment (1,95 g/kg of feed). There was high significant difference between treatments.

4.3.2 Sodium bicarbonate consumption
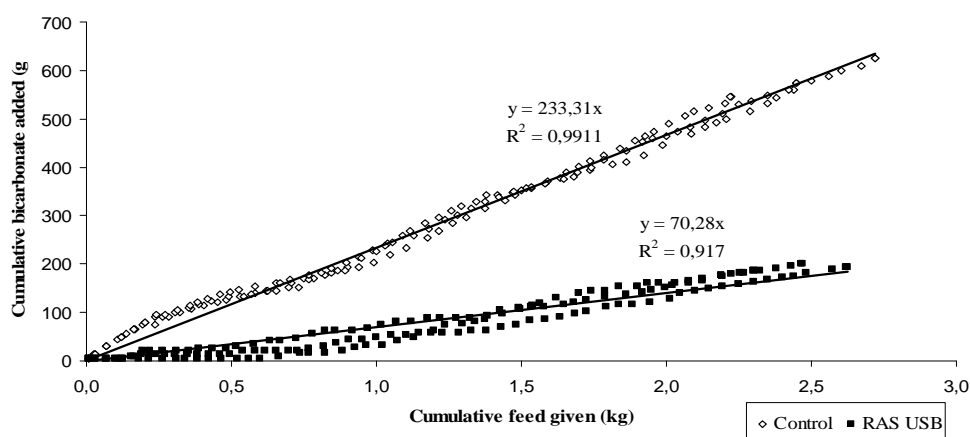
Figure 13: Cumulative sodium bicarbonate consumption (g) per kilogram of total cumulative feed for both treatments (p= 0.00, from the ANCOVA analysis)

Cumulative bicarbonate consumption per cumulative feed given is presented in figure 13. It evolutes proportionally being for control treatment 233,3 g/kg of feed and for RAS USB treatment 70,3 g/kg of feed, with highly significant difference between treatments.

4.4 Fish feed composition

The analyzed composition of the feed given to turbot during the experimental period is presented on table 10. There are presented values for g of nutrient per kg of feed and g of nutrient per kg of feed on a dry matter basis. Comparing these values with the values given by the company that manufactured this feed (see table 3), we are able to see some differences in content.

For protein content on dry matter basis we found higher values of protein (58,5%) in relation to the company information (54%), but a lower content regarding to g of protein per kg of feed (52,6%). The same could be observed for fat content. On Phosphate content, both dry matter basis and total feed basis were lower than the value given by the manufacturer. Carbohydrates were higher on dry matter basis (11,7%) but the value measured for total feed basis were the same compared to the value given by the company (10,5%).

The crude protein to gross energy ratio for this feed was 24,99 mg/KJ, it was calculated as crude protein/gross energy.

Table 10: Nutrient composition of the feed used during the experimental period, presents on a total feed weight basis and feed dry matter basis.

Component	g/kg of feed	g/kg of feed (DM basis)
Dry matter	899,90	-
Ash	92,20	102,46
Crude protein	526,92	585,54
Fat	175,19	194,7
Energy	21,09 Kj/g	23,43 Kj/g DM
Phosphate	11,19	12,44
Acid insoluble ash (AIA)	0,808	0,898
Carbohydrates	105,58	117,33

4.5 Body fish composition

The body composition of fish was measured at the start and at the final of the experiment, and the values for dry matter, ash, crude protein, fat and phosphate are presented in table 11 as % of nutrient per fresh body weight, being the value for energy presented in KJ/g of fresh body weight.

There were no significant differences in body composition between both treatment for all the measured values.

On table 10, it is also presented the value of retention of nutrient in fish. These values are presented in g of nutrient/$kg^{0,8}$/day, it can be found highly significant differences of retention values for dry matter and crude protein (p<0,01). For energy retention, there was no significant difference when tested with statistical T-test, although when used the statistical Mann-Whitney test it showed significant difference. This difference is due to the power of the test.

The values for COD retention were obtained from the calculations shown in chapter 2. Fish showed 10,4±1,16 % and 12,84±1,11 % for control and RAS USB treatment respectively, a significant difference was found between the treatments (p<0,05).

Table 11: Values for fish body composition (% fresh body weight, mean±S.D.) at start and final of the experiment. Retention of nutrient on fish body is also presented ($g/kg^{0,8}$/day, mean±S.D.). P<0,01 indicates highly significant differences and p<0,05 indicates significant differences between treatments (T-student test)

	Day 0	Day 54			Retention		
		Control	**RAS USB**	**P-value**	**Control**	**RAS USB**	**P-value**
Dry matter	25,99	26,74±0,31	26,54±0,14	0,77	1,2±0,10	1,5±0,10	**0,01**
Ash	3,45	3,65±014	3,44±0,20	0,56	0,2±0,02	0,18±0,04	0,65
Crude protein	16,72	16,15±0,35	16,49±0,10	0,09	0,55±0,04	0,89±0,03	**0,00**
Fat	5,90	6,59±0,33	6,44±0,32	0,21	0,31±0,11	0,40±0,12	0,35
Energy (KJ/g)	6,36	6,35±0,22	6,31±0,24	0,49	25,04±3,58 (KJ/ $kg^{0,8}$/day)	36,81±5,64 (KJ/ $kg^{0,8}$/day)	**0,07***
Phosphate	0,61	0,58±0,03	0,58±0,03	0,91	0,02±0,004	0,03±0,007	0,54

* showed no significant difference with T-student test, but with Mann-Whitney test there was significant difference

4.6 Digestibility

The apparent digestibility coefficients (ADC) for nutrients in the diet are presented in table 12. There are presented three different calculations for ADC. Since ADC is calculated by the relation between the amount of feed given and the correspondent faeces collected, errors are associated with this calculation, affecting ADC values of dry matter, ash and phosphate. The formulas are presented in chapter 2.

ADC without correction was lower than ADC with correction and real digestibility for dry matter, ash and phosphate. For the same nutrient, it was also lower the ADC with correction in relation with real digestibility. Regarding to the rest of feed nutrients, the real digestibility was higher than the values of apparent digestibility coefficient.

The efficiency of waste recovery was evaluated by calculating AIA recovery, which was 0,58±0,13.

Table 12: Apparent digestibility coefficient (% ADC) of the nutrients in the diet, with a correction for the minerals in the system water (15 ppt) and corrected for feed waste.

	ADC without correction (%)	ADC with correction (%)	Real Digestibility (%)
Dry matter	68,76±5,62	78,76±3,90	86,2±4,17
Ash	13,1±10,11	41,92±10,01	47,6±12,36
Phosphate	38,5±11,69	41,8±10,66	45,3±12,03
Crude protein	82,31±3,05	82,31±3,05	89,7±3,90
Energy	79,04±3,84	79,04±3,84	86,3±4,28
Fat	87,12±3,19	87,12±3,19	94,1±3,98
Carbohydrates	28,63±14,62	28,63±14,62	36,5±10,31

4.7 Energy budget

Results of energy balance are presented in table 13, values are presented in energy per metabolic body weight per day. For gross energy intake (**GE**), fecal energy loss (**FE**), digestible energy (**DE**), branchial urinary energy (**BUE**) and metabolize energy intake (**ME**) there was no significant difference between treatments.

For retained energy (**RE**) and energy requirement for maintenance (**MEm**), we found significant difference between treatment (p<0,05), being energy retention higher and the energy requirement for maintenance lower for turbot in RAS USB treatment. The last one was calculated using two different k_g, when MEm was calculated with k_g of 0,7 it show to be lower than when it was calculated with k_g of 0,8.

The energy loss by heat (**H**) showed significant difference between treatments using T-student test.

Table 13: Energy budget for turbot in both treatments (control and RAS USB), presented as mean±S.D. P<0,05 indicates significant difference between treatments (T-student test)

		Control (KJ/kg^{0,8}/day)	RAS USB (KJ/kg^{0,8}/day)	P-value
Gross Energy intake		77,92±9,62	78,41±6,35	0,93
Fecal Energy loss		62,33±7,70	62,72±5,08	0,93
Digestible Energy		15,59±1,93	15,69±1,27	0,93
Branchial Urinary Energy		3,95±0,48	3,97±0,32	0,93
Metabolize Energy intake		58,38±7,22	58,74±4,76	0,93
Retained Energy		24,02±1,82	38,19±7,98	**0,04**
Heat		34,36±7,86	20,55±3,42	**0,049**
Energy requirement for maintenance	K_g 0,7	27,15±8,12	9,09±5,76	**0,036**
	K_g 0,8	26,41±8,04	13,90±8,04	**0,039**
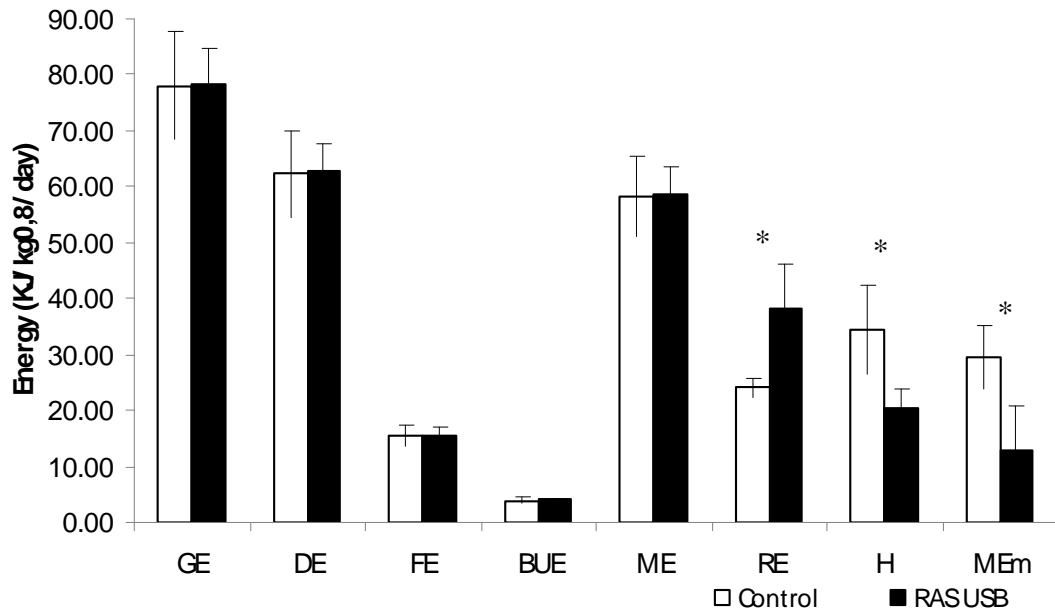
Figure 14: Energy balance for turbot in both treatments (control and RAS USB), MEm was calculated with k_g of 0,8. * indicates significant difference between treatments (p<0,05, T-student test)

The figure above (figure 14), represent table 12 in a graphic presentation with MEm is presented with k_g of 0,8. As it was said before there is no significant difference between treatments, except for RE, H and MEm.

4.8 Nitrogen mass balance

The nitrogen balance for both treatments is presented in table 13. Values are presented in g of nitrogen per kg of feed given and % of nitrogen per feed given. The results showed no significant difference in the amount of nitrogen given for both treatments (**N feed**).

Turbot in RAS USB treatment showed higher nitrogen (**N growth**) retention (41,67%) compared with turbot in control (26,45%), with highly significant difference (p<0,01). Regarding to nitrogen in water discharge (**N discharge dissolved**), it was higher in control system (59,89%) compared with RAS USB treatment (17,26%), highly significant difference (p<0,01).

For nitrogen accumulated in the systems (**N accumulated system**) it was negative in both treatments, being higher the accumulation in RAS USB treatment (-1,67%) compared with control treatment (-6,28%), with significant difference between treatments.

For the nitrogen, that can not be explained by the mass balance (**N unexplained**) we found significant differences between treatments (p<0,05), being higher for control systems (9,65%) compared with RAS USB treatment (-0,08%).

Table 14: Nitrogen mass balance components in g of N/kg of feed given and in percentage for both treatments P<0.05 indicates a significant difference between treatments (t test)

	Control		RAS USB		P-value
	g/kg of feed	%	g/kg of feed	%	
N feed	84,3±0,03	100,00	84,31±0,00	100,00	-
N growth	22,3±2.01	26,45	35,13±2,01	41,67	0,00
N removed USB	-	-	28,28±0,96	33,54	-
N discharge solid	8,7±0,00	10,29	-	-	-
N discharge dissolved	49,3±2,71	59,89	14,55±3,16	17,26	0,00
N accumulated USB	-	-	7,82±3,70	9,27	-
N accumulated system	-5,5±0,64	-6,28	-1,41±1,72	-1,67	0,02
N unexplained	9,5±3,53	9,65	-0,07±2,39	-0,08	0,03

4.8 Phosphorus mass balance

The phosphorus mass balance for both treatments is presented in table 15. Values are presented in g of phosphorus per kg of feed given and % of phosphorus per feed given. The results showed no significant difference in the amount of phosphorus given by feed for both treatments (**P feed**).

Comparing the phosphorus retained in turbot (**P growth**), there was no significant difference between treatments (p>0,05).

As in nitrogen mass balance, phosphorus accumulation in the systems (**P accumulated system**) were higher for RAS USB treatment (29,15%) compared with control (-3,63%), showing highly significant difference (p<0,01) between treatments. Regarding to phosphorus in the water discharge (**P water discharge**), it was lower for RAS USB treatment (13,42%) compared with control (21,90), with highly significant difference (p<0,01).

For the amount of phosphorus that could not be explained (**P unexplained**) we did not find any significant differences between treatments (p>0,05).

Table 15: Phosphorus mass balance components in g of P/kg of feed given and in percentage for both treatments P<0.05 or P<0,01 indicates a significant and highly significant difference, respectively between treatments (t test)

	Control		RAS USB		P-value
	g/kg of feed	%	g/kg of feed	%	
P feed	11,2±0,01	100,00	11,20±0,02	100,00	-
P growth	5,7±1,27	50,57	6,14±1,27	54,82	0,70
P discharge solid	3,8±0,00	33,76	-	-	-
P water discharge	2,5±0,19	21,90	1,50±0,21	13,42	0,00
P accumulated USB	-	-	0,29±0,00	2,62	-
P accumulated system	-0,4±0,11	-3,63	3,26±0,11	29,15	0,00
P unexplained	-0,3±1,66	-2,60	0,00±1,07	-0,02	0.81

4.8 Chemical Oxygen Demand (COD) mass balance

The COD mass balance for both treatments is presented in table 16. Values are presented in g of COD per kg of feed given and % of COD per feed given. The results showed no significant difference in the amount of COD given by feed for both treatments (**COD feed**).

COD retained in turbot (**COD growth**) was higher for RAS USB treatment (47,4%) compared with control (37,8%), showing significant difference ($p<0,05$). Also significant difference ($p<0,05$) was observed for turbot respiration (**COD respiration fish**), but in this case it was lower for RAS USB treatment (32,7%) than control treatment (53,0%). The values for COD on solid discharge (**COD discharge solid**) from the USB reactor were obtained from sludge discharge, as to the values for the control, they were obtained from faeces collection.

For COD that could not be explained in the mass balance (**COD unexplained**), we found significant differences between systems ($p<0,05$), being higher for RAS USB treatment (14,6%) than control (-2,7%).

Table 16: Chemical Oxygen Demand mass balance components in g of COD/kg of feed given and in percentage for both treatments P<0.05 indicates a significant difference, respectively between treatments (t test)

	Control		RAS USB		P-value
	g/kg of feed	**%**	**g/kg of feed**	**%**	
COD feed	1279,7±0,74	100,0	1279,9±2,29	100,0	-
COD growth	483,4±65,82	37,8	607,0±32,77	47,4	0,04
COD respiration fish	644,45±82,07	50,36	384,72±90,05	30,07	0,02
COD discharge solid	145,64±0,08	11,38	11,3±0,24	0,9	-
COD respiration USB			95,5±3,23	7,5	-
COD accumulated USB			-39,6±18,13	-3,1	-
COD unexplained	6,18±51,85	0,5	220,99±85,57	17,6	0,018

5. Discussion

The aim of this study was to determine the role of an Upflow Sludge Blanket denitrifying reactor, in growth performance of *P. maxima* and establish the efficiency of it determining the minimum water exchange required to maintain nitrate-N concentration at 150 mg/L. This was evaluated based on fish, system and USB reactor performance, also water quality using the following parameters: growth, FCR, feed intake and digestibility, fish composition, TAN, NO_2-N, NO_3-N concentration, sludge composition.

5.1 Fish performance

The feed intake in this study showed no significant differences between the two treatments, being equal to 2,2 ± 0,21g/day/fish. It is lower compared with Hop (2008) experiment, that consisted on determining turbot performance on low pH RAS (pH 5,7) and normal pH RAS (pH 7,5). In this experiment the same systems were used, and for the control treatment the feed intake increased during the experiment from 2,67±0,32 to 4,20±0,29 g/day/fish. In the same experiment, the measurements of TAN, nitrite-N were, in general, lower compared with the same measurements in this experiment. As to nitrate-N the concentration started lower at 90 mg/L reaching 150 mg/L at the final of the experiment. Comparing feed intake in both experiments with the concentrations of TAN, nitrite and nitrate, it is observed that for our experiment feed intake was lower with higher concentration of these nitrogenous compounds. This might be interpreted as a negative influence on feed intake by these nitrogenous compounds, since our fish started with a higher concentration of this compounds. Although NO_3-N concentration on Hop (2008) experiment increased as well as the feed intake (regarding to the control treatment), but just during a short period. After the ending of the experiment, feed intake decrease, possible indicating a negative effect of nitrate in feed intake (Heinsbroek, personal communication).

Growth was significantly higher in the treatments with USB reactor. The specific growth rate for fish in control systems was significantly lower (0,46±0,04 %BW/day) in relation with fish on RAS USB treatment (0,63±0,04 %BW/day). Growth rate for RAS USB treatment was in agreement with what was obtained in other study with turbots, 0.65% BW/day (Mallekh et al., 1998), in this experiment turbot was reared on flow-through system,

indicating that fish reared under on RAS with USB reactor had similar growth rates, this is a possible advance regarding to growth retardation compared with flow-through systems. Hop (2008) found a specific growth rate of 0.73 %BW/d in their control system. It was better than what was obtained in both treatments; this might be explained by the difference in water quality, since nitrogenous compounds like TAN, nitrite and nitrate are generally higher in our experiment.

Regarding to feed conversion, fish from RAS USB treatment had significant lower FCR 0,68±0,04 g/g compared with fish in control (0,92±0,08 g/g), also conversion efficiency was significantly higher for RAS USB treatment. Comparing with literature this FCR of fish in control is considered to be in the same order as Burel et al. (1996) who found values of 0,8-0,9 and Mallekh et al (1998) who found values of FCR ranging 0,96. This difference with the literature might be due to FCR calculation, since we used values of feed eaten and not feed given, it may also be related to the cultivation technique. In relation to fish in RAS USB treatment, FCR was lower, comparing with Hop (2008) experiment, with values of 0,85 g/g for the pH treatment and 0,71 g/g for the control treatment, this control treatment of Hop being the same system for our experiment, showed a lower FCR in relation to our experiment (also our control treatment).

By the energy budget we could find significant differences between treatments in relation to retained energy, heat and energy requirement for maintenance. For fish in RAS with USB, retained energy was higher and energy requirement for maintenance as well as heat were lower, as it is shown in table 13. Due to the fact that there was no significant difference in feed intake, one possible explanation for a better growth can be based on efficiency using feed as source of energy for growth.

The values for energy requirement for maintenance were based on using a k_g of 0,7 and 0,8. The efficiency of protein and lipid deposition for other species is obtained by regression of retained energy versus digestible energy intake (Lupatsch et al., 2003). On this experiment such a regression could not be done due to lack of sufficient values.

However, for a lean fish as turbot, a k_g value of 0,7 is more likely. With k_P (efficiency of protein deposition) of 0.55 (Heinsbroek et al., 2007, Lupatsch et al., 2003) and k_L (efficiency of lipid deposition) of 0.9 (with a fat rich feed, as we had, (Heinsbroek et al., 2007)), it is possible to calculate the k_g with the values of retention for protein and fat. This is done by knowing the amount of energy retained by fat and protein using the mean heat values to convert protein in energy of 23,6 KJ/g of protein and to convert fat in energy of 39,5 KJ/g

of lipid (Brafield and Llewellyn, (1982) in Azevedo, Milgen et al. (2005)). This amount of protein and fat energy retained is multiplied by k_P and k_L, respectively and divided by the total amount of energy retained of protein and fat, giving a k_g of 0,72 for control treatment and kg of 0,7 for RAS USB treatments, it is therefore concluded that a k_g of 0,7 is the best guess to this fish species. A study made by Lupatsch et al (2003) found k_g values for three saltwater species (European sea bass, gilthead sea bream and white grouper) ranging from 0,65 to 0,69.

Few literature is available for values of energetic requirements for maintenance for turbot and flatfish in general. We found values in literature for other saltwater species, for instance European sea bass (*Dicentrarchus labrax*) showing MEm rounding 43,6 to 50,9 KJ DE/$kg^{0,8}$/day at 19-26º C and 25º C respectively (Lupatsch et al 2001; Peres and Oliva-Teles, 2005), gilthead sea bream (*Sparus aurata*) showing values of 55,8 KJ DE/$kg^{0,8}$/day (Lupatsch et al 1998), yellowtail (*Seriola quinqueradiata*) 62,7 KJ DE/$kg^{0,8}$/day (Watanabe et al., 2000) and white grouper (*Epinephelus aeneus*) 34,05 KJ DE/$kg^{0,8}$/day (Lupatsch et al 2003).

In the study of Pichavant et al. (2000), juvenile turbot were kept under darkness deprived of feed for 7 days, and the oxygen consumption was calculated. With this value we could estimate the energy requirement for maintenance by the oxycaloric equivalent (13,6 KJ.g/O_2). With this calculation we obtained a value for MEm of 14,4 KJ/$kg^{0,8}$/day, this value is lower compared with the values for this experiment for fish in control and also lower compared with other species, Eel: 21 KJ/$kg^{0,8}$/day (Heinsbroek et al.,2007,2008), Catfish, 21 KJ/$kg^{0,8}$/day (Hogendoorn, 1983). Comparing this value with our obtained values for MEm we can observe that, for the control treatment either with a Kg of 0,7 or 0,8 they are above the value obtained by Pichavant et al. (2000) for fasting turbot, and for the RAS USB treatment both are below the values obtained by Pichavant et al. (2000). With this study, it seems like MEm calculated with a k_g of 0,7 is out of range being to low (9,09±5,76 KJ/$kg^{0,8}$/day). MEm with a k_g of 0,8, is still lower compared with values for fasting turbot in Pichavant et al. (2000), although it appears to be a more reasonable values than MEm with a k_g of 0,7. This could be explained by the fact that, the values of kg were estimated and not calculated, for further studies it could be interesting to calculate these values, even though it is observed that fish reared under USB reactor had lower energetic requirement for maintenance.

One possible explanation for lower values found in this experiment for both treatments compared with literature can be based in turbot benthic life style. Flatfish have markedly lower metabolic rate than pelagic fish (Mallekh and Lagardére, 2002), the values from literature described above show that fish with pelagic life style have higher MEm compared

with benthic fish, for instance yellowtail, a physiologically well adapted pelagic fish, has higher MEm than white grouper, a species that is more frequently found on benthic areas. Literature of MEm is not abundant for flatfish making the comparison of values obtained in this study more difficult.

As referred before, turbot growth in RAS with the USB reactor was significantly higher compared with control, this is in agreement with values for MEm. With less energy needed for basal metabolism, more energy can be used for growth. MEm is strongly related with body size and water temperature but since there were no significant differences between these parameters in the two treatments, other explanation must be found. MEm is also influenced by stress, when fish are in adverse environment they spend more energy to overcome the stressing factor, for instance by the activation of the immune response. By visual inspection fish seemed calm in both treatments, with no visible differences.

As to heat production, we found significant differences between treatments. The heat produced by fish, calculated with the energy budget, showed to significantly higher for fish in the control treatment ($34,36\pm7,86$ KJ/kg^{0,8}/day) compared with fish in RAS USB treatment ($20,55\pm3,42$ KJ/kg^{0,8}/day). As it was stated by Kleiber (1975) (in Bureau, Kaushik et al. 2003), that the metabolic rate is the rate at which heat is liberated from the individual as a result of internal body reactions, so less heat produced correspond to a lower metabolic rate thus less energy expenditure. This is in agreement with the result that we had, since the heat produced by fish in RAS USB was lower, corresponding to a lower MEm and a higher growth rate. The heat produced is related to feed intake, thus higher feed intake will require higher metabolism to transform feed in energy, since feed intake did not differ between treatments and with the described above, we can suggest that the USB reactor had influence on the heat produced.

For turbot body composition, there were no significant differences between treatments in all nutrient content in fish body. We found for values of crude protein $\pm16\%$, fat $\pm6\%$, energy ±6 KJ/g and phosphate $\pm0,58\%$ for both treatments using a commercial feed with the following composition: 58,6% of protein; 19,4% of fat; 23,4 KJ/g of energy; 1,2% of phosphate all in a dry matter basis.

In the experiment of Regost and Arzel et al. (2003) they investigated the replacement of fish oil by vegetables oil, with the feed (crude protein 57%/dry matter, crude fat 16,6%/dry matter; gross energy 23,5 KJ/g/dry matter) more similar to the feed given to our fish, the author found the following body composition: 17% protein; 4,8% fat both in % of wet weight and 6,1 KJ/g. The same author but in other study (Regost and Arzel et al., 2001) comparing

different contents in fat, obtained the following values for body composition of turbot: 17% protein; 3,8% fat, both in % wet weight; 5,6% KJ/g in this study the protein and fat content of the experimental diet was higher (61,3%/ dry matter crude protein 21.4%/ dry matter crude fat). The same author obtained in other study (Regost and Arzel et al., 1999) with the control feed (49% crude protein; 12% crude fat; 20,7 KJ/g energy; 1,6% phosphorus all in dry matter basis), values for body composition of 16% protein; 2,72% fat; 0,62% phosphorus all in % of wet weight and 4,9KJ/g for energy content.

From literature, we can observe that the percentage of fat was lower than the obtain in our experimental fish, even in the experiment of Regost and Arzel et al., (2001) where the fat content of the experimental diet was higher. Although the fat content of the experimental feed of Regost and Arzel et al., (2001), was obtained in a higher part form wheat gluten, a carbohydrate (16,4% of wheat gluten and 14% fish oil), Bureau and Kaushik (2002) stated that lipid efficiency of deposition is lower when lipids are synthesized from carbohydrates, also carbohydrates have a lower digestibility (in our experiment ADC of carbohydrates was the lowest, table 12) thus lipid deposition was lower in Regost and Arzel et al., (2001) experiment.

The values of protein, for one experiment mentioned above (Regost and Arzel et al., 1999) fish showed a similar value in body composition besides the feed had a lower content of protein compared with our feed, although values of protein content in fish body seems to be in range with literature. For energy and phosphorus content also seems to be in the same range with the literature described. Though for the experiment of Regost and Arzel et al., (1999) the energy content in fish was lower (4,9 KJ/g compared with 6 KJ/g in our fish), this is due to energy content in feed being lower than the feed used in our experiment.

As to retention significant differences were found for dry matter, crude protein, COD and energy, being all higher for RAS USB treatment. The last one only showed significant difference when applied Mann-Whitney test, considering the lower accuracy of this test this should not happen, but taking into account that this experiment was made with only three replicates and we obtained a high standard deviation this significant difference can be accepted, comparing the differences in protein retention between treatments and the differences in energy retention. In our experiment, we found for protein retention values of 26,9±2,17% and 45,7±3,0% of protein intake for control and RAS USB respectively, for energy 32,9±6,06% and 46,9±2,89% of energy intake for control and RAS USB respectively and for fat 58,5±12,99% and 68,8±4,57% of fat intake for control and RAS USB respectively,

the last one presents a high standard deviation due to replicate number 6, presented high values of fat content in relation to the other treatments.

Comparing with literature, values in our experiment are in general higher than the values found by Regost and Arzel et al. (2001) and Regost and Arzel et al. (1999) which presented for protein 29,6% and 41% and for energy 17% and 28,5%, respectively (as % of intake). Regarding to protein, the retention of the control treatment is in the same range but retention for RAS USB is considerably higher, it is also higher the energy retention but in this case for both treatments. The higher retention of protein, can be explained by a higher content in protein in our feed, compared with the feed used by Regost and Arzel et al., (1999), although the same feed was given to both treatments, and fish in control showed a lower retention. This indicates a higher efficiency in protein deposition by fish under USB reactor. As to fat retention, values obtained by Regost and Arzel et al., (1999) were considerably lower (18,8%) compared with both treatments, this is related to the higher fat content in our feed.

As to fish digestibility, three different methods are presented. The first, ADC without correction was obtained by simply applying the ADC formula (3.8.3). ADC with correction, it was calculated due to the influence of minerals in water altering the values of ash and phosphate, dry matter was also corrected, this was made due to the large amount of water content in faeces (weight of wet faeces: 346,8±54,8 g; weight of dry faeces:27,9±4,3 g). As to real digestibility, it was calculated taking into account the amount of feed waste since when we collected faeces the feed waste could not be separated, decreasing the value of digestibility. As it can be seen in table 12 that values increased from ADC without correction to real digestibility.

In the studies mentioned above ADC was also calculated with indirect method, using other markers (chromic oxide and yttrium oxide). Values of ADC for protein, ADC for fat and ADC for energy found were, respectively: 95,%, 94,4% and 86,1% for the experiment of Regost and Arzel et al., (2001). 94%, 77,6% (of starch) and 71% for the experiment of Regost and Arzel et al (1999). 96%, 95% and 90 % Regost and Arzel et al.,(2003). Compared with our values for digestibility, the closest to the literature are the values of real digestibility: 89,7% protein, 94% fat and 86,3% energy. Regarding to phosphorus, digestibility found in our experiment was low (45,3%) compared with the one found in Regost and Arzel et al (1999) of 71%, although the differences in digestibility and fish body composition are not so

large compared with our experiment (phosphate body composition in our experiment: 0,58%; Regost and Arzel et al., (1999): 0,62%), this could be explained by a sampling error.

5.2 Water quality

In this experiment the water quality was kept in the programmed range. The nitrate-N concentration was initially higher than planned, but this was corrected with an extra water exchange.

Regarding to water temperature, it was constant during the experimental period - around 17ºC- this is in agreement with the temperature ranges found by Boeuf et al (1998). This author considered the optimum temperature to be between 16-19ºC, and Mallek et al (1984) found the highest growth rate at 18ºC, in intensive farming conditions. As referred in the literature review, an increase in temperature will increase the energy required for basal metabolism. Since there was no difference between treatments, the influence of temperature can not be considered.

The same can be said for oxygen levels. During the experiment, it was remained stable at ± 7,4 mg/L, in the study of Pichavant et al. (2000) the higher increase in mean body weight was found at 7,2 mg/L oxygen concentration. Mallekh and Lagardére (2002) studied the maximum uptake of oxygen by turbot and the authors concluded that, there is no gain in increase oxygen concentration to supersaturation in intensive turbot farming.

As regards to water salinity, it kept constant around 15 ppm. Gaumet et al (1995) found the best growth rate and FCR with salinity ranging from 10 to 19 ppm for juvenile turbots. The same was found for pH values. pH in both systems was kept in the best range for turbot growth (Hop, 2008).

On the subject of nitrogen compounds, ammonia-N was high in the beginning of the experiment considering the values for chronic effect found by Person-Le-Ruyet et al (1997), although for that experiment, juvenile turbots were used (14 to 104g) also the authors found that bigger fish have lower tolerance to ammonia. In our experiment, ammonia-N stabilized around 0,35 mg/L. In general, concentration of TAN and nitrite were higher for the RAS USB treatment, this could be explained by lower efficiency of the nitrifying bacteria. This lower efficiency may be related to the competition between nitrifying and denitrifying bacteria in the trickling filter (Eding, personal communication), since a decrease of biomass of the USB reactor was observed, part of this bacterial biomass could end up in the trickling filter.

The nitrite concentration showed higher values for the RAS USB treatment, this is an interesting observation since this treatment showed the best growth rates and FCR, considering nitrite to be the nitrification product with higher toxicity. Huguenin and Colt (2002) suggested 0,1 mg/L as a threshold for aquatic animals, on our experiment we found much higher values for both treatments. This higher nitrite concentrations could be explained by passive denitrification, like it was said in chapter 1.4.3, nitrite production occur in microsites of the RAS where the oxygen level is low and a carbon source is available, making this type of denitrification one disadvantage producing nitrite instead of nitrogen gas. Also this nitrite production could be due to incomplete denitrification, resulting from a low C/N ration in the USB reactor (Tiedje, 1990 in Timmons and Ebeling 2007), derived from a low C/N ration in the feed and also depending on fish conversion efficiency. In addition on the nitrite concentration during the experiment, an increase was observed after the second week, this could be related with the extra water exchange with the new water that entered the system influencing the nitrification. It could also be explained by the fact that, when TAN concentration was high at the beginning, the higher removal rate could result in higher diffusion of nitrite from the bacteria biofilm in the trickling filter, slightly increasing the concentration in the water (Eding and Kamstra, et al., 2006).

As to nitrate, the concentration was kept around 150 mg/L during all experimental period. At the beginning of the experiment (the first week) the nitrate concentration was higher than the expected, so an extra water exchange was made to reduce the nitrate concentration. Significant difference was observed on week 5 and 8, but considering the overall period it is reasonable to believe that there were no effect of this significant differences. As it is referred in the literature review, it is believed that high nitrate concentrations could affect negatively fish growth, studies on species like Medaka fish showed low tolerance to relatively low concentrations of nitrate, Shimura et al (2004) found growth supression with concentrations between 100 and 125 mg/L, but little is known about the effect of nitrate in more comonly cultured species.

In an earlier experiment in the GRRAS project, Heinsbroek (Personal communication) compared different systems also using two treatments, one (**A**) with USB reactor and nitrate concentration of 50 mg/L the other (**B**) without an USB reactor and with 180-240 mg/L of nitrate, on both treatments the water exchange was low (150 L/kg of feed). This experiment showed a higher growth for treatment **A** giving the idea of the possible effect of nitrate in growth, although this experiment and our experiment can not be compared because it was

made with different batch of fish in different systems. But it suggested a negative influence of nitrate and/or a positive influence of the denitrification reactor, on turbot growth.

In our experiment, the nitrate-N concentration was kept at 150 mg/L for both treatments and we found significant differences in growth rates and feed conversion in the treatment with the denitrification reactor, this suggest that nitrate has influence growth but it may not be the most important factor regarding to growth retardation in RAS, since for a concentration of 150 mg/L it was found significant differences between treatments, being the growth rates higher for treatment with USB reactor.

As to concentration of orthophosphate in the culture water, we found significant difference on the first week and second week forward until the end of the experimentl we found higlhy significant differences being higher on RAS USB treatment, increasing the concentration of PO_4-P from $8,8\pm0,69$ mg/L until $24,95\pm0,38$ mg/L on day 54, compared with control system which was kept between $9,12\pm0,74$ and $7,12\pm0,32$ mg/L (day 0 and 54 respectively). This increase of dissolved phosphorus occurred due to the bacterial community in the USB reactor. A study made by Barak and Cytryn et al. (2002), with a zero-discharge RAS showed that the PO_4-P concentration did not exceed 15 mg/L, this is considerable lower compared with our experiment, although this experiment the denitrification occurred on aerobic and anaerobic conditions, they showed that under aerobic conditions concentrations of total phosphate were higher, under anaerobic conditions this concentrations were lower and dissolved phosphate concetrations were higher (Barak and Cytryn et al. 2002). Indicating that a higher concentration of dissolved phosphat should occur under anaerobic denitrification, like in our experiment.

This high concentration of PO_4-P observed in the RAS USB treatment did not affect fish growth, since turbot had good growth rates and feed conversions. Although on an environmental point of view, this high concentration of PO_4-P could be prejudicial since aquatic systems (freshwater and some saltwater systems) are normally limited by phosphorus. With the increase of the limiting parameter enhancement of algal growth occur, provoking eutrophization.

At the middle of the experimental period, a small difference was observed in the water color between treatments, in RAS USB treatment was slightly darker (yellow-brownish) compared with control treatment. The differences in color might be due to humic acids formation. As referred in chapter 2.2.4 humic acids are large, complex organic molecules which are produced by degradation of organic matter (Hubbard et al, 2002). The same author

described a study where humic acids were tested to capture steroid pheromones in aqueous environments, reducing significantly the ability of fish to detect these chemical signals. This study may explain the removal from water solution of the stress corticosteroid hormone, cortisol. Fridell et al (2007), made a trial infecting Atlantic salmon with a virus and observed that water cortisol concentrations were greatly elevated in infected groups, compared to uninfected groups, reflecting elevated plasma cortisol concentrations, relating an increase in cortisol levels in water with an increae of stress. There is the possibility that humic acids are capable to bind the this type of hormones, reducing stress levels in fish concomitant with a lower energy requirement for maintance having more energy to use in growth. Even though as it was said before, no difference was seen it fish behavior between treatments, evaluated by visual observation.

Although trials made with stress induced using cortisol did not always show depression in growth, in some studies inconsistency relating cortisol to depression of growth were observed (Van Weerd and Komen 1998).

As it is referred in chapter 1.3.7, the presence of signaling substances emanated by fish, could alter their development. Evidence of crowding factors released into the water by fish, with esters characteristics, could also be related to a higher growth in fish under an USB reactor, since the formation of humic acids could inhibit the chemical stimuli that this steroids normally induce. Showed in chapter 1.3.7, where fish that were not growing properly due to larger fish in the same tank, when transfer to other aquaria they started to grew normally. It is interesting for further studies, to analyze the difference in size of the same system, thus larger fish could induce a slower growth in smaller fish, but the possible effect of humic acids could disable this type of signaling, increasing the growth rate. Being cheaper and easier than analyzing steroids in water, although with higher risk of errors.

Also humic acids are believed to bind ions and with toxic metals such as cadmium, removing them from water no longer affecting fish (Meinelt et al, 2001).

5.3 System performance

The system efficiency was evaluated with the cumulative water exchange, the cumulative nitrogen production and removal, cumulative orthophosphate-P production and cumulative sodium bicarbonate consumption.

Regarding to water exchange, the aim for this experiment was to maintain a water exchange of 30 L/kg of feed in RAS USB treatment and 300 L/kg of feed in control treatment. As it was shown in figure 10, the water exchange for control treatment was respected being 299,8 L/kg of feed. In the other treatment (RAS USB) a water exchange of 58,9 L/kg feed was needed to keep the nitrate-N concentration at 150 mg/L. This was due to the low C/N ratio in the feed that was going to the USB reactor as waste. It was referred in chapter 3 that C/N ration is one of the factors that influence denitrification performance, with the low C/N ration found in feed given to experimental turbots, the nitrate removal was lower than the expected, so the water exchange needed to be higher to maintain nitrate at 150 mg/L. This low C/N ration result in the accumulation of intermediate products of denitrification as it was referred above.

Considering the higher growth for fish with USB reactor, and the water exchange for both treatments, it is plausible that the denitrification reactor instead of just removing the growth inhibition factor (**GIF**), could also produce GIF neutralizing substances (for instance humic acids, as referred before). Considering both water exchange it was expected that with higher water exchange the GIF were also removed from the culture water. On the other hand, if the USB reactor is viewed as water exchange, and considering that the flow through the USB reactor was around 1750 L/kg of feed (GRRAS, 2008), the water exchange would have been higher than the control treatment (300 L/kg of feed). This fact would explain the higher growth rates for fish in recirculation system with a low water exchange, considering that this low water exchange would provoke an accumulation of GIF in the cultures water.

As to nitrogen production, is in agreement with what was observed in Hop (2008) experiment, he found nitrate production of 45,6 g of NO_3-N/kg of feed. On our experiment the mainly composition of nitrogen compounds was of nitrate-N, since it is the end product of nitrification. We found for the control treatment 44,2 g of N/kg of feed and for RAS USB treatment 42,1 g of N/kg of feed, showing no significant differences.

The dissolved phosphate production, showed significant differences between treatments, being for control treatment 1,9 g of PO_4-P/kg of feed and for the RAS with USB reactor 4,8 g of PO_4-P/kg of feed, this is due to bacteria in the denitrification reactor. The increase of phosphorus concentration in water could have a beneficial effect, as referred in chapter 2.2.3, the requirement of phosphorus is normally met by the feed due to his low concentration in water although as it was said for nitrate about it difficulty to be absorbed by the gills due to its size, for phosphorus considering that is even bigger than nitrate its

permeability should be lower (Heinsbroek, personal communication). It must also be taken into account that phosphorus levels were not a limiting factor in feed.

The sodium bicarbonate used to maintain the pH was significantly higher for control systems compared with RAS with USB, this is due to denitrification were for each mole of nitrate removed, one equivalent alkalinity is produced (van Rijn, Tal et al. 2006).

5.4 Mass balances

5.4.1 Nitrogen balance

The nitrogen balance showed that fish from systems with USB reactor retained an amount of nitrogen significantly higher from the fish in the control systems, with 41,7% and 26,5% respectively. These differences support the fact that USB reactor had a positive effect on growth by removing GIF or releasing GIF neutralizing substances. As to water discharge, a higher amount of nitrogen was discharge in the control systems 59,9% compared with the systems with USB reactor, 17,3%, this indicate a positive influence on an environmental basis reducing the amount of nitrogen in the culture waste water.

Several studies had referred that elevated concentrations of NH_4^+, NO_2^- and NO_3^-, derived from human activities, can stimulate or enhance the development of primary producers (micro and macroalgae), contributing to the well known phenomenon of eutrophication of aquatic ecosystems (Camargo and Alonso, 2006).

Regarding to accumulation of nitrogen in both systems, significantly differences were found, with -6,3% for the control systems and -1,7% for RAS with USB reactor. The values for both treatments were found to be negative, this indicate that the amount of nitrogen that was found in the beginning of the experiment was higher than the values found at the end, this could be explained by the extra water refreshment than was made on the second week, analyzing the figures 6 and 8 we can observe that after this extra refreshment the concentration of the nitrogenous compounds decreased. It could also be explained by a loss of biomass during the experiment and as it was referred before a low feed C/N ration could explain this loss as all the organic input into the USB reactor was "consumed", reducing the amount of nitrogenous compounds. Although for control treatment, that operated without an USB reactor, the decrease of nitrogenous compounds was also observed.

The nitrogen unexplained, is based in all the errors in measurements and sampling that could occur and had to be taken into account, but it is not possible to say with accuracy where this errors occurred, also it is need to take into account the passive denitrification, like it was referred in chapter 1.4. This passive denitrification probably took place in the flask that was collecting faeces for the USB reactor, but further studies must be done to prove its influence on the overall balance. Also, at the end of the experiment wile the experimental set up was been cleaned, we observed sludge on the out let pipe of the fish tank, this bacteria community may also contribute to the passive denitrification, contributing to higher nitrite concentrations in this treatment.

5.4.2 Phosphate balance

Regarding to phosphate balance, no significant difference was found in phosphate retention by fish in both treatments. An interesting remark regarding this retention value is that, comparing it with values for phosphate digestibility (45,3±12,03% for RD) the retained phosphorus was higher than the digestibility of it (control: 50,57%; RAS USB: 54,82% for phosphate retention). This could indicate a possible uptake of phosphorus from the water, although for control treatment that has a lower PO_4-P concentration also presented higher value of retention compared with digestibility. Also these values of digestibility were calculated based on faeces collected from the fish of the control treatment, thus the digestibility in fish from RAS USB could be higher for phosphate.

As to phosphate in water discharge a significant difference was found between treatments, being 21.9% for control treatment and 13,4% for RAS USB treatment. This indicates again a positive effect on the utilization of an USB reactor on an environmental basis, as referred before RAS with an USB reactor also had lower levels of nitrogen in water discharge and in spite of a significantly difference in production of orthophosphate in these systems (figure 9), the phosphate discharge is lower compared with the control treatment. Nitrogen and phosphorus are the main factors causing eutrophization on the world water sources, reducing their discharge to the environment is a main issue on aquaculture development and these results shows a good via towards the sustainable development of recirculation aquaculture.

As to accumulation of phosphate in the systems, there were significant differences between treatments, being higher in RAS USB treatment (29,2%) compared with control

treatment (-3,6%). Despite the RAS USB treatment had lower concentrations of phosphate in the water discharge, it had considerable high concentrations of PO_4-P accumulated in the system. As it was referred above (chapter 5.2) this could present an environmental problem, since the solubilized phosphate accumulated in the system is discharged in the environment with the water discharge, although the non solubilized phosphate in the water discharge was lower being a possitive environmental remark. As to unexplained phosphate there was no significant difference between treatments.

5.4.3 COD balances

As to COD balances, we found significant differences on the retention of COD in fish between treatments, being 47,4% for RAS USB and 37,8% for control treatment. On the other hand, COD from fish respiration shows to be significantly higher for fish in the control treatment (53,0%) compared with fish in RAS USB treatment (32,7%). The first one can be explained by the same reason for nitrogen retention in fish; USB has a positive effect on growth, possibly by removing GIF or releasing GIF neutralizing substances. The second one could be explained bioenergetically, has a higher need for maintenance requirement, thus a higher respiration rate. This was calculated with the heat produced by fish, that was calculated with the oxycaloric equivalent of 13,36 KJ.g/O_2. As it was said above, fish from RAS USB showed a lower MEm, which is in agreement with values of COD from fish respiration, thus higher MEm is related with higher need of oxygen for metabolic processes.

As to COD unexplained, significant differences were found between treatments being 14,6% for RAS USB and -2,7% for control treatment

5.5 Conclusions

The main goal of this experiment was to evaluate the growth performance of *P. maxima* reared with an upflow sludge blanket reactor. The following conclusions were made:

- *P. maxima* showed a better performance when reared with an USB reactor, combined with a low water exchange and constant nitrate concentration. This better performance could be explained by higher feed conversion efficiency and a lower energy requirement for maintenance, translated in a higher growth rate.

- Considering constant nitrate concentration and lower water exchange, it can be said that USB reactor had a positive influence on growth performance of *P. maxima*, either by removing possible Growth Inhibition Factors, or releasing substances that could neutralize them.

- The USB reactor was not able to maintain the nitrate concentration at 150 mg/L with a water exchange of 30L/kg of feed, this had to be increased to 58,9 L/kg of feed. This fact was explained either by the low C/N ration in *P. maxima* feed or its conversion efficiency.

- The incorporation of an USB reactor on a recirculation system, seems to have beneficial effects for sustainable development of aquaculture, reducing water expenditure and nitrogenous compounds in water discharge without affecting growth performance of fish. However, care is needed with the increase of the dissolved phosphorus in the culture water.

Future recommendations:

- It would be interesting to look more carefully to the reason with energy requirements for maintenance were lower for fish reared under USB action.

- Also understand the influence not only of nitrate on fish performance, but also humic acids and orthophosphate on water quality and fish performance.

- For further uses of USB reactor, an economical study should be made to assess its viability.

6. References

Aboutboul, Y., R. Arviv, et al. (1995). "Anaerobic treatment of intensive fish culture effluents: volatile fatty acid mediated denitrification." Aquaculture **133**(1): 21-32.

Arbiv, R. and J. van Rijn (1995). "Performance of a treatment system for inorganic nitrogen removal in intensive aquaculture systems." Aquacultural Engineering **14**(2): 189-203.

APROMAR (2008), La Acuicultura Marina de Peces en España, Asociason Empresarial de Productores de Cultivos Marinos

Aubin, J., E. Papatryphon, et al. (2006). "Characterisation of the environmental impact of a turbot (Scophthalmus maximus) re-circulating production system using Life Cycle Assessment." Aquaculture **261**(4): 1259-1268.

Azevedo, P.A., Van Milgen, J., Leeson, S., Bureau, D.P., (2005). Comparing efficiency of metabolizable energy utilization by rainbow trout (Oncorhynchus mykiss) and Atlantic salmon (Salmo salar) using factorial and multivariate approaches. Journal of Animal Science 83 (842-851)

Barak, Y., E. Cytryn, et al. (2003). "Phosphorus removal in a marine prototype, recirculating aquaculture system." Aquaculture **220**(1-4): 313-326.

Boeuf, G., D. Boujard, et al. (1999). "Control of the somatic growth in turbot." Journal of Fish Biology **55**(SUPPL. A): 128-147.

Bureau, D. P., S. J. Kaushik, et al. (2002). Bioenergetics. Fish Nutrition (Third Edition). San Diego, Academic Press**:** 1-59.

Burel, C., Person-Le Ruyet, J., Gaumet, F., Le Roux, A., Sévère, A., Boeuf, G., 1996. Effects of temperature on growth and metabolism in juvenile turbot. Journal of Fish Biology 49, 678-692. Chelon labrosus. Mar. Environ. Res. 45, 357–365.

Cal, R. M., S. Vidal, et al. (2006). "Growth and gonadal development in diploid and triploid turbot (Scophthalmus maximus)." Aquaculture **251**(1): 99-108.

Camargo, J. A. and Á. Alonso (2006). "Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment." Environment International **32**(6): 831-849.

Camargo, J. A., A. Alonso, et al. (2005). "Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates." Chemosphere **58**(9): 1255-1267.

Coles, C. A. and R. N. Yong (2006). "Humic acid preparation, properties and interactions with metals lead and cadmium." Engineering Geology **85**(1-2): 26-32.

Confente, F., A. El M'Rabet, et al. (2008). "The pineal complex of Senegalese sole (Solea senegalensis): Anatomical, histological and immunohistochemical study." Aquaculture **285**(1-4): 207-215.

Evaluation of the performance of turbot (*Psetta maxima* L.) in RAS with and without an Upflow Sludge Blanket reactor.

Deviller, G., C. Aliaume, et al. (2004). "High-rate algal pond treatment for water reuse in an integrated marine fish recirculating system: effect on water quality and sea bass growth." Aquaculture **235**(1-4): 331-344.

Deviller, G., O. Palluel, et al. (2005). "Impact assessment of various rearing systems on fish health using multibiomarker response and metal accumulation." Ecotoxicology and Environmental Safety **61**(1): 89-97.

Eding, E. H., A. Kamstra, et al. (2006). "Design and operation of nitrifying trickling filters in recirculating aquaculture: A review." Aquacultural Engineering **34**(3): 234-260.

Dosdat, A., F. Servais, et al. (1996). "Comparison of nitrogenous losses in five teleost fish species." Aquaculture **141**(1-2): 107-127.

FAO Fisheries department, 2006. State of the world aquaculture 2006. FAO Fisheries technical paper, 500, 134p.

FAO, 2008. Website: http://www.fao.org/fishery/culturedspecies/Psetta_maxima

Fishbase website: www.fishbase.org

Fraser, K. P. P., A. D. Rogers, et al. (2007). Protein Metabolism in Marine Animals: The Underlying Mechanism of Growth. ADVANCES IN MARINE BIOLOGY, Academic Press. **Volume 52:** 267-362.

Fridell, F., K. Gadan, et al. (2007). "Effect of hyperoxygenation and low water flow on the primary stress response and susceptibility of Atlantic salmon Salmo salar L. to experimental challenge with IPN virus." Aquaculture **270**(1-4): 23-35.

Gaumet, F., G. Boeuf, et al. (1994). "Effects of environmental water salinity on blood acid-base status in juvenile turbot (Scophthalmus maximus L.)." Comparative Biochemistry and Physiology Part A: Physiology **109**(4): 985-994.

Hamlin, H. J. (2006). "Nitrate toxicity in Siberian sturgeon (Acipenser baeri)." Aquaculture **253**(1-4): 688-693.

Hamlin, H. J., B. C. Moore, et al. (2008). "Nitrate-induced elevations in circulating sex steroid concentrations in female Siberian sturgeon (Acipenser baeri) in commercial aquaculture." Aquaculture **281**(1-4): 118-125.

Hara, T. J. (1975). "Olfaction in fish." Progress in Neurobiology **5**(Part 4): 271-335.

Heinsbroek, L. T. N., P. L. A. Van Hooff, et al. (2007). "Effects of feed composition on life history developments in feed intake, metabolism, growth and body composition of European eel, Anguilla anguilla." Aquaculture **267**(1-4): 175-187.

Heinsbroek, L. T. N. and A. Kamstra (1990). "Design and performance of water recirculation systems for eel culture." Aquacultural Engineering **9**(3): 187-207.

Heinsbroek, L.T.N., Tijssen, P.A.T., Flach, R.B. & De Jong, G.D.C (1993) Energy and nitrogen balance studies in fish. In: Fish Nutrition in Practice (Kaushik, S. & Luquet, P. eds.), pp. 375±389. INRA, Paris, France.

Heinsbroek, L.T.N., van Thoor, R.M.H. & Elizondo, L.J. (1990) The effect of feeding level on the apparent digestibilities of nutrients and energy of a reference diet for the European eel, Anguilla anguilla L. and the African catfish, Clarias gariepinus (Burchell). Proc. 3rd Int. Symp. Feeding and Nutr. in Fish, August 28 September 1, 1989, Toba, Japan, pp. 175±188.

Heylen, K., B. Vanparys, et al. (2006). "Cultivation of Denitrifying Bacteria: Optimization of Isolation Conditions and Diversity Study." Appl. Environ. Microbiol. **72**(4): 2637-2643.

Hochberg, R. B., S. L. Pahuja, et al. (1991). "Steroidal fatty acid esters." The Journal of Steroid Biochemistry and Molecular Biology **40**(4-6): 577-585

Hogendoorn, H. (1983). "Growth and production of the African catfish, Clarias lazera (C. & V.): III. Bioenergetic relations of body weight and feeding level." Aquaculture **35**: 1-17.

Hop, J., 2008. Effects of low pH on production of turbot (Psetta maxima) in recirculating aquaculture systems. MSC thesis, Wageningen university, 45p.

Hubbard, P. C., E. N. Barata, et al. (2002). "Possible disruption of pheromonal communication by humic acid in the goldfish, Carassius auratus." Aquatic Toxicology **60**(3-4): 169-183.

Imsland, A. K., A. Foss, et al. (2001). "The interaction of temperature and salinity on growth and food conversion in juvenile turbot (Scophthalmus maximus)." Aquaculture **198**(3-4): 353-367.

Imsland, A. K. Foss, A.. Stefansson, O. (2001). "Variation in food intake, food conversion efficiency and growth of juvenile turbot from different geographic strains." Journal of Fish Biology **59**(2): 449-454.

Ip, Y. K., S. F. Chew, et al. (2001). Ammonia toxicity, tolerance, and excretion. Fish Physiology, Academic Press. **Volume 20:** 109-148.

J A Grøttum, M. S., T Sigholt (1997). "Effect of oxygenation, aeration and pH control on water quality and survival of turbot, Scophthalmus maximus (L.), kept at high densities during transport." Aquaculture Research **28**: 159-164.

Jobling, M. Fish bioenergetics. London : Chapman & Hall, 1994. 294p

Jönsson, E., V. Johansson, et al. (2003). "Central nervous system actions of growth hormone on brain monoamine levels and behavior of juvenile rainbow trout." Hormones and Behavior **43**(3): 367-374.

Kroupova H. Machova J. and Svobodova, Z. (2005) Nitrite influence on fish: a review, Vet. Med. Czech 50 (11), pp. 461–471.

Kroupova, H., J. Machova, et al. (2008). "Effects of subchronic nitrite exposure on rainbow trout (Oncorhynchus mykiss)." Ecotoxicology and Environmental Safety **71**(3): 813-820.

Labatut, R. A. and J. F. Olivares (2004). "Culture of turbot (Scophthalmus maximus) juveniles using shallow raceways tanks and recirculation." Aquacultural Engineering **32**(1): 113-127.

Lall, S. P., E. H. John, et al. (2003). The Minerals. Fish Nutrition (Third Edition). San Diego, Academic Press**:** 259-308.

Evaluation of the performance of turbot (*Psetta maxima* L.) in RAS with and without an Upflow Sludge Blanket reactor.

Lall, S. P. and L. M. Lewis-McCrea (2007). "Role of nutrients in skeletal metabolism and pathology in fish -- An overview." Aquaculture **267**(1-4): 3-19.

Lupatsch, I., Kissil, G.Wm., Sklan, D., Pfeffer, E., (1998). Energy and protein requirements for maintenance and growth in gilthead sea bream (Sparus aurata L.). Aquaculture Nutrition 4 (165-173).

Lupatsch, I., Kissil, G.Wm., Sklan, D., (2001). Optimization of feeding regimes for European sea bass Dicentrarchus labrax: a factorial approach. Aquaculture 202 (289-302).

Lupatsch, I., Kissil, G.Wm., Sklan, D., (2003). Comparison of energy and protein efficiency among three fish species gilthead sea bream (Sparus aurata), European sea bass(Dicentrarchus labrax) and white grouper (Epinephelus aeneus): energy expenditure for protein and lipid deposition. Aquaculture 225 (175-189).

Mallekh, R., J. P. Lagardère, et al. (1998). "Variability in appetite of turbot, Scophthalmus maximus under intensive rearing conditions: the role of environmental factors." Aquaculture **165**(1-2): 123-138.

Mallekh R. and Lagardère J.P., (2002) Effect of temperature and dissolved oxygen concentration on the metabolic rate of the turbot and the relationship between metabolic scope and feeding demand, Journal of Fish Biology. 60, pp. 1105–1115

Meinelt, T., R. C. Playle, et al. (2001). "Interaction of cadmium toxicity in embryos and larvae of zebrafish (Danio rerio) with calcium and humic substances." Aquatic Toxicology **54**(3-4): 205-215.

Mommsen, T. P., T. W. Moon, et al. (2001). 8. Hormonal regulation of muscle growth. Fish Physiology, Academic Press. **Volume 18:** 251-308.

Person-Le Ruyet, J., K. Pichavant, et al. (2002). "Effects of O2 supersaturation on metabolism and growth in juvenile turbot (Scophthalmus maximus L.)." Aquaculture **205**(3-4): 373-383.

Pichavant, K., J. Person-Le-Ruyet, et al. (2000). "Effects of hypoxia on growth and metabolism of juvenile turbot." Aquaculture **188**(1-2): 103-114.

Pierce, R.H., Weeks, J.M., Prappas, J.M., 1993. Nitrate toxicity to five species of marine fish. J.World Aquacult. Soc. 24, 105–107.

Power, D. M., L. Llewellyn, et al. (2001). "Thyroid hormones in growth and development of fish." Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **130**(4): 447-459.

Peres, H., & Oliva-Teles A. (2005). Protein and energy metabolism of European seabass (Dicentrarchus labrax) juveniles and estimation of maintenance requirements. Fish Physiology and Biochemistry 31 (23-31)

Reid, P.M. Wilkinson, A.E. Tipping, E. and Jones, M.N. (1991) Aggregation of humic substances in aqueous media as determined by light-scattering methods. J. Soil Sci. 42, pp. 259–270.

Roales, R. R., (1981) The effect of growth inhibition factors on lipid content on the total lipid content of the zebrafish *Brachydanio verio* (Hamilton-Buchanan). Journal of Fish Biology 18,723-728

Rousseau, K., N. Le Belle, et al. (2001). "Pituitary Growth Hormone Secretion in the Turbot, a Phylogenetically Recent Teleost, Is Regulated by a Species-Specific Pattern of Neuropeptides." Neuroendocrinology **74**(6): 375-385.

Evaluation of the performance of turbot (*Psetta maxima* L.) in RAS with and without an Upflow Sludge Blanket reactor.

Ruyet, J. P.-L., R. Galland, et al. (1997). "Chronic ammonia toxicity in juvenile turbot (Scophthalmus maximus)." Aquaculture **154**(2): 155-171.

Schneider, O., V. Sereti, et al. (2007). "Kinetics, design and biomass production of a bacteria reactor treating RAS effluent streams." Aquacultural Engineering **36**(1): 24-35.

Shimura, R. Ma, Ying X, Shunji, Kenichi Ijiri, Uchiyama, Nagaoka and Minoru (2004) "Nitrate Toxicity on Visceral Organs of Medaka Fish, Oryzias latipes : Aiming to Raise Fish from Egg to Egg in Space", Biology Science Space, Vol. 18, pp.7-12

Solomon D.J. (1977) A review of chemical communication in freshwater fish. Journal Fish Biology. (1977) 11, 363-376

Sumpter, J. P. (1992). "Control of growth of rainbow trout (Oncorhynchus mykiss)." Aquaculture **100**(1-3): 299-320.

Tarlera S., Denner E. (2003) Sterolibacterium denitrificans gen. nov., sp. nov., a novel cholesterol-oxidizing, denitrifying member of the b-Proteobacteria International Journal of Systematic and Evolutionary Microbiology. 53, 1085–1091

Timmons, M.B. and Ebeling, J.M. (2007) Recirculating Aquaculture, Cayuga Aqua Ventures, Ithaca, NY, USA.

Tchobanoglous G, Burton FL, Stensel HD. 2007. Wastewater engineering: treatment and reuse

van Rijn, J., N. Fonarev, et al. (1995). "Anaerobic treatment of intensive fish culture effluents: digestion of fish feed and release of volatile fatty acids." Aquaculture **133**(1): 9-20.

van Rijn, J., Y. Tal, et al. (2006). "Denitrification in recirculating systems: Theory and applications." Aquacultural Engineering **34**(3): 364-376.

Van Weerd, J. H. and J. Komen (1998). "The effects of chronic stress on growth in fish: a critical appraisal." Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology **120**: 107-112.

Wang, S. and C. N. Mulligan (2009). "Enhanced mobilization of arsenic and heavy metals from mine tailings by humic acid." Chemosphere **74**(2): 274-279.

Watanabe K., Ura K., Yada T., Kiron V., Satoh S. & Watanabe T. (2000) Energy and protein requirements of yellowtail for maximum growth and maintenance of body weight. Fisheries Science 66, 1053–1061

Wood, A. W., C. Duan, et al. (2005). Insulin-Like Growth Factor Signaling in Fish. International Review of Cytology, Academic Press. **Volume 243:** 215-285.

Zumft, W. G. (1997) Cell biology and molecular basis of. denitrification. Microbiol. Mol. Biol. Rev., 61:533-616.