Grupo de Acuicultura y Biodiversidad

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OPTIMIZATION OF NITRIFYING BIOFILTERS FOR AQUACULTURE

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RESUMEN

Los sistemas de recirculación en acuicultura (definidos como circuitos cerrados con mínimo o nulo intercambio del agua de cría) son unos sistemas de producción acuícola con mucho potencial, en particular debido a la independencia respecto a un suministro continuo de agua limpia y la capacidad de controlar los parámetros de calidad de agua incluyendo temperatura y nivel de oxígeno disuelto. Sin embargo, su aplicación se ve frenada por los mayores costes de inversión, la complejidad técnica y el nivel de conocimiento necesario para lograr el correcto funcionamiento de los equipos de purificación del agua. La optimización de uno de estos equipos, el biofiltro nitrificante, encargado de la eliminación del amonio generado por los peces como consecuencia de su metabolismo proteico, tóxico en altas concentraciones, es el objetivo de la presente tesis. Para ello se realizó una serie de experimentos donde se evaluó el rendimiento de biofiltros nitrificantes de percolación (expresado como tasa de eliminación amoniacal pero también prestando atención a la concentración de amonio máxima y mínima en el tanque) en función de diferentes características como son la carga de amonio en el influente del biofiltro, el tipo de material filtrante, la temperatura y la carga hidráulica. La combinación de parámetros (material filtrante, carga hidráulica y temperatura) que originó el mejor rendimiento del biofiltro fue seleccionada para diseñar los biofiltros usados en un experimento de cría de doradas (*Sparus aurata*) (especie seleccionada puesto que su rango de temperatura óptimo es equivalente a las temperaturas que originaron una mayor tasa de eliminación de amonio) en las mismas instalaciones. Durante este experimento, se reevaluó el rendimiento del biofiltro, sujeto a la influencia de la materia orgánica, en función del tipo de pienso y la estrategia de alimentación usados para la cría de la dorada.

Los biofiltros piloto se instalaron en sistemas independientes y la evaluación de su rendimiento se realizó en primer lugar mediante el cálculo de la diferencia entre una cantidad de amonio añadida y la observada tras 24 horas de la adición amoniacal, cálculos que se realizaron por cuadruplicado por cada una de la combinación de parámetros influyentes en las tasas de eliminación de amonio. La reevaluación de las tasas de eliminación durante el experimento de cría de peces se realizó restando a la cantidad de amonio excretada (estimada en los mismos sistemas para los mismos peces), la cantidad de amonio presente en el tanque a las 24 horas después de la alimentación. Los peces fueron alimentados con tres piensos diferentes (un control, uno formulado a base de harina de pescado y uno formulado sin harina de pescado) y el

pienso fue suministrado mediante tres estrategias diferentes de alimentación (a saciedad, utilizando comederos automáticos y utilizando comederos de auto-demanda).

De la primera serie de experimentos se concluyó que, de los materiales filtrantes probados, uno de ellos (MECHpro® rings) fue responsable de rendimientos muy inferiores mientras que el uso de los materiales filtrantes restantes provocó un rendimiento muy similar. También se concluyó que la temperatura tuvo un efecto muy importante, pues bajas temperaturas (entre 15 y 18 ºC) originaron rendimientos bajos para cualquier combinación restante de parámetros por inactivación de las bacterias nitrificantes, mientras que a temperaturas altas el efecto de los diferentes parámetros, como la carga hidráulica, fue más significativo. Respecto a esta última, se observó un rendimiento claramente inferior a una carga hidráulica baja (4 m^3/m^2 h), así como rendimientos similares a las cargas hidráulicas restantes (8 m^3/m^2 h y 11 m^3/m^2 h).

Las tasas de eliminación de amonio de los biofiltros nitrificantes durante su utilización para la cría de doradas fue generalmente menor que en las pruebas de determinación del efecto de diferentes configuraciones en el rendimiento de biofiltros. Esto fue atribuido a la presencia de materia orgánica, pero también a la menor carga de amonio ligada a unas excreciones de amonio total bajas. Se observó asimismo que el pienso vegetal implicó una pérdida de rendimiento y también se concluyó que una determinada estrategia de alimentación (utilización de comederos de auto-demanda) produjo rendimientos superiores respecto a las restantes estrategias, tanto en tasa de eliminación amoniacal como en concentración de amonio en agua. El valor máximo de concentración de amonio fue el más bajo de todos los experimentos realizados, incluyendo los resultados de las pruebas de evaluación del efecto los parámetros operacionales en la tasa de eliminación de amonio.

Por tanto, los resultados de la presente tesis demuestran la importancia del diseño de los parámetros operacionales para el correcto funcionamiento de biofiltros de nitrificación percoladores. En concreto, se observaron diferencias muy significativas en el rendimiento respecto a determinados parámetros como el material filtrante o la temperatura. Otros parámetros, como la carga de amonio, demostraron conducir a mayores tasas de eliminación de amonio al amentar su cantidad, pero también originaron picos de concentración de amonio más elevados, lo que cuestiona su conveniencia en el correcto funcionamiento de los sistemas. Por otro lado, se manifiesta la importancia de parámetros nutricionales como el pienso o la estrategia de

alimentación en el funcionamiento de biofiltros nitrificantes y por ello deben ser considerados para el diseño y operación de sistemas de recirculación en acuicultura.

SUMMARY

Recirculating aquaculture systems (defined as closed circuits where rearing water exchange is reduced to a minimum) show a great potential for fish production, in particular due to the independence of a continuous clean water supply that they provide, as well as the capacity of management of the water quality parameters, including temperature and dissolved oxygen. Nevertheless, their global use as a fish production system faces the disadvantages of higher initial investment costs, the technical complexity and the level of expertise required to achieve the correct operation of the water purification equipment. The optimization of one of those units, the nitrifying biofilter, which removes the ammonia generated by fish as a consequence of their protein metabolism but toxic to them in high concentration, is the subject of the present thesis. The performance (determined as ammonia removal rate but also taking into account the maximum and minimum concentration of N-TAN in the tank water) of a determinate type of these biofilters, the trickling filter, was evaluated according to different characteristics known as process parameters, such as the biofilter influent ammonia load, filter medium type, water temperature or hydraulic loading. The combination of those process parameters that led to the best results in biofilter performance was used to design biofilters for use in a pilot-scale rearing trial of gilthead sea bream (*Sparus aurata*), whose optimal growth temperature range is equivalent to the temperatures which led to the highest mean ammonia removal rate. During that experiment, carried out on the same facilities as the performance trials, the performance of the biofilter was re-evaluated, now subject to the influence of organic matter, depending on the diet and the feeding strategy used to feed the gilthead sea bream.

The pilot biofilters were placed in independent systems and their efficiency was determined by the calculation of the difference between a certain quantity of ammonia added and the observed in the tank water after 24 hours of addition. Those calculations were performed four times for every combination of parameters relevant to the ammonia removal rates. The re-evaluation of the removal rates during the fish rearing trial was calculated as the difference between the daily excretion of the fish (estimated with the same fish on the same systems) and the quantity of ammonia present in the tank water 24 hours post-feeding. Fish were fed three different diets (a control diet, a formulated diet with fishmeal and a formulated diet without fishmeal) y the feed was supplied by three different feeding strategies (to satiation, using automatic feeders and using auto-demand feeders).

The first batch of experiments showed that, from the filter media assessed, one of them (MECHpro® rings) was responsible of low performance in general, while the ammonia removal rates were comparable with the use of the remaining filter media. It was also concluded that the temperature had a very important effect as low temperatures (between 15 and 18ºC) led to low ammonia removal independent of every combination of the remaining process parameters due to the inactivation of nitrifying bacteria, while the effect of other process parameters, such as the hydraulic loading, was more significant at high temperatures. In regard to this parameter, an inferior efficiency was observed at a low hydraulic loading (4 m^3/m^2 h) compared to the remaining hydraulic loadings (8 m^3/m^2 h y 11 m^3/m^2 h), although both values led to similar efficiencies.

The mean ammonia removal rate of the nitrifying biofilters during operation in the gilthead sea bream rearing trial was generally lower than in the trials regarding the effect of the combination of process parameters on biofilter performance. This was attributed to the presence of organic matter, but also to the lower ammonia load consequence of low total ammonia excretion. In addition, it was observed that the plant meal based diet led to a loss of performance and that a determinate feeding strategy (use of auto-demand feeders) led to higher biofilter performances compared to the remaining feeding strategies, both in ammonia removal rate and in N-TAN concentration in the tank water. The maximum N-TAN concentration was the lowest across every experiment, including the results of the trials regarding the effect of the combination of process parameters on biofilter performance.

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In conclusion, the results of the present thesis demonstrate the importance of the process parameters design for the correct operation of nitrifying trickling filters. Concretely, very significant differences in performance were found considering process parameters such as filter media or temperature. Other parameters, such as the ammonia load, led to higher ammonia removal rates when it was increased, but also originated higher ammonia concentration peaks, which questions its convenience in the correct functioning of the systems. On the other hand, it is also brought to light that certain aspects of fish production such as the diet composition or feeding strategy influence the performance of nitrifying filters and therefore should be considered for the design and operation of recirculating aquaculture systems.

RESUM

Els sistemes de recirculació en aqüicultura (definits com circuits tancats amb un intercanvi mínim o nul d'aigua) són uns sistemes de producció aqüícola amb molt de potencial, en concret degut a la independència respecte a un subministrament continuat d'aigua neta i a la capacitat de controlar els paràmetres de qualitat de l'aigua on s'inclouen la temperatura i el nivell d'oxigen dissolt. Tanmateix, la seua aplicació es veu frenada pels majors costos d'inversió inicial, la seua complexitat tècnica i el nivell de coneixement necessari per a aconseguir el correcte funcionament dels equips de filtració de l'aigua. La optimització d'un d'aquests equips, els biofiltres nitrificants, encarregat de la eliminació de l'amoni generat pels peixos com a conseqüència del seu metabolisme proteic que es torna tòxic en altes concentracions, es l'objectiu de la present tesi. Per a tal funció es va elaborar una sèrie d'experiments on es va avaluar el rendiment de biofiltres nitrificants de percolació (expressat com taxa d'eliminació amoniacal però tenint en compte la concentració d'amoni màxima i mínima) en funció de diferents característiques como són la càrrega d'amoni en l'influent del biofiltre, el tipus de material filtrant, la temperatura i la càrrega hidràulica. La combinació de paràmetres (material filtrant, temperatura i càrrega hidràulica) que va originar el major rendiment del biofiltre fou seleccionada per a dissenyar els biofiltres a utilitzar per a l'experiment de cria de dorades (*Sparus aurata*) en les mateixes instal·lacions, ja què el seu rang de temperatura òptim és coincident amb les temperatures que varen original la major taxa d'eliminació d'amoni. Durant aquest experiment, es va re-avaluar el rendiment del biofiltre, subjecte a la influència de la matèria orgànica, en funció del tipus de pinso i la estratègia d'alimentació per a la cria de la dorada.

Els biofiltres pilot es varen instal·lar en sistemes independents i l'avaluació del seu rendiment es va realitzar en primer lloc mitjançant el càlcul de la diferència entre una quantitat d'amoni afegida i la observada 24 hores després de l'adició, càlculs que es varen fer per quadruplicat per qualsevol combinació de paràmetres influents en les taxes d'eliminació d'amoni. La re-avaluació de les taxes de eliminació durant l'experiment de cria de peixos es va realitzar restant a la quantitat d'amoni excretada (estimada en els mateixos sistemes amb les mateixos peixos) la quantitat d'amoni present en l'aigua del tanc 24 hores després de l'alimentació. Els peixos varen ser alimentats amb tres pinsos diferents (un control, un formulat amb farina de peix i un formulat sense farina de peix) i el pinso va ser subministrat mitjançant tres estratègies d'alimentació (a sacietat, usant comeders automàtics i usant comeders d'auto-demanda.

De la primera sèrie d'experiments es va concloure que, dels materials filtrants provats, un d'ells (MECHpro® rings) fou responsable de rendiments molt inferiors, mentre que l'ús dels restants materials filtrants va produir rendiments molt similars entre ells. També es va concloure que la temperatura va tindre un efecte molt important perquè baixes temperatures (entre 15 i 18 ºC) conduïren a baixos rendiments amb independència de la combinació dels altres paràmetres, degut a la inactivació de les bactèries nitrificants. Per una altra banda, amb altes temperatures, l'efecte de paràmetres com la càrrega hidràulica sobre les taxes d'eliminació d'amoni fou més significatiu. Respecte a aquesta última, es va observar un rendiment clarament inferior a una càrrega hidràulica baixa (4 m^3/m^2 h) respecte a les restants càrregues hidràuliques (8 m^3/m^2 h y 11 m^3/m^2 h) que varen originar similar rendiments entre elles.

El rendiment dels biofiltres nitrificants durant la seua utilització per a la cria de dorades fou generalment menor que en les proves de determinació dels efectes de diferents configuracions en la taxa d'eliminació d'amoni. Això fou atribuït a la presència de matèria orgànica, però també a la menor càrrega d'amoni lligada a unes excrecions d'amoni total baixes. Es va observar tanmateix que el pinso vegetal va implicar una pèrdua de rendiment i també es va concloure que una determinada estratègia d'alimentació (utilització de comeders d'auto-demanda) va produir rendiments superiors respecte a les restants estratègies, tant en taxa d'eliminació amoniacal como en concentració d'amoni en l'aigua. El valor màxim de concentració d'amoni fou el mes baix de tots els experiments realitzats, incloent els resultats de les proves d'avaluació del efecte dels paràmetres operacionals en la taxa d'eliminació d'amoni.

En conclusió, els resultats de la present tesi demostren la importància del disseny dels paràmetres operacionals per al correcte funcionament de biofiltres nitrificants de percolació. En concret, es varen observar diferències molt significatives en el rendiment respecte a determinats paràmetres com el material filtrant o la temperatura. Altres paràmetres, com la càrrega d'amoni, demostraren conduir a taxes d'eliminació majors al augmentar la seua quantitat, però també originaren pics de concentració d'amoni més alts. També es posa de manifest que certs aspectes de la producció aqüícola com la composició del pinso o la estratègia d'alimentació tenen una influència directa en el rendiment de biofiltres nitrificants i per tant deuen ser considerats per al disseny i operació de sistemes de recirculació en aqüicultura.

TABLE OF CONTENTS

ABBREVIATIONS

RAS: Recirculating Aquaculture Systems
N: Nitrogen
P: Phosphorus
TAN: Total ammonia nitrogen
AOB: Ammonia oxidizing bacteria
NOB: Nitrite oxidizing bacteria
ANOVA: Analysis of variance
ANCOVA: Analysis of co-variance
SEM: Standard error of the mean
FM: Fish meal diet (100% of fish meal)
VM: Plant meal diet (0% of fish meal)
CON: Control diet
SGR: Specific growth rate
FCR: Feed conversion ratio
COD: Chemical oxygen demand
BOD: Biological oxygen demand
OM: Organic matter

GENERAL INTRODUCTION

1. INTENSIFICATION OF AQUACULTURE AND ENVIRONMENTAL IMPACT

Aquaculture is one of the food producing industries that has expanded the most during the last 30 years (Boyd and McNevin, 2015; Natale et al., 2013). As capture fishery is unable to increase due to exploitation (Crab et al., 2007), aquaculture is considered the only viable source to provide seafood to meet the increasing demand. The naturally high efficiency of aquaculture to produce protein-rich food has been perceived as an opportunity for satisfying food demand especially in developing countries (Boyd and McNevin, 2015). Aquaculture production has increased continuously to this day, reaching 80.03 million tons in 2016, excluding water plants (FAO 2018) (Figure 1.1), whilst capture fisheries are stagnant between 88 and 95 million tons (90 million tons in 2016). In addition, it must be considered that a significant amount of fisheries' production is destined to supply fish meal and oil to aquaculture companies (Natale et al., 2013).

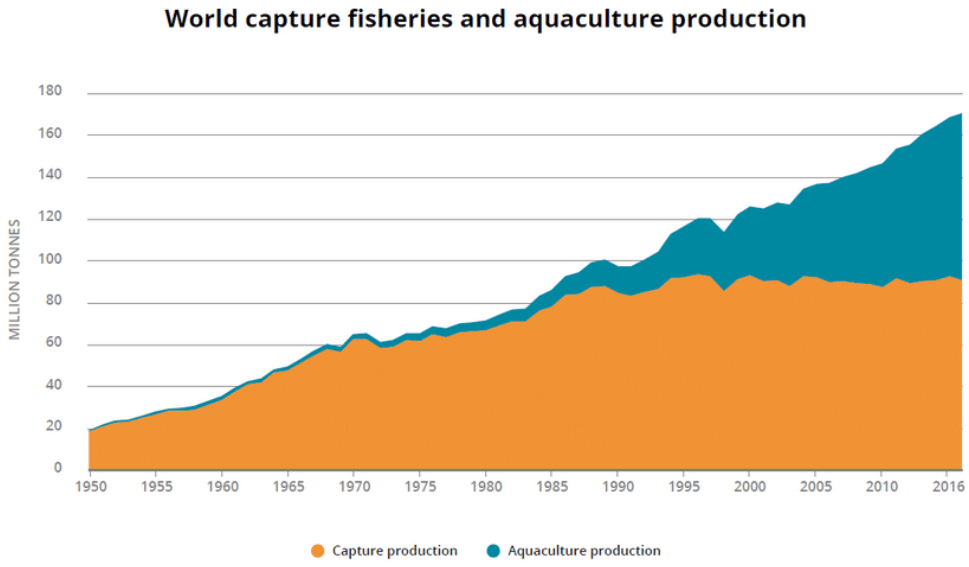

Figure 1.1. Production of fish by capture fisheries and aquaculture companies from 1950 to 2016 (latest data geared). Does not include aquatic plants and other aquatic animals such as alligators or crocodiles. Source: The State of the World Fisheries and Aquaculture, FAO (2018).

With the current status of wild fish stocks and risk of overfishing pointed out by experts, non-governmental organizations and consumers in general, aquaculture is often remarked as a more sustainable food producing industry than capture fishery but, as any other industry in the world, is not, however, free of producing environmental impacts. The perception of aquaculture as a "environmentally friendly" industry is crucial as population that is already aware of the risk of overfishing is unlikely to increase the demand of reared fish if their production is not clearly more sustainable than capture fishery. Intrinsic

environmental impacts of aquaculture are the depletion of marine feedstuffs to produce fish feed ingredients (Karapanagiotidis, 2014) and the release of nutrient-rich effluents containing mostly nitrogen and phosphorus, as well as organic matter consisting in uneaten food and faeces (Herath and Satoh, 2015). Environmental impacts of an aquaculture facility as well as their magnitude are very varied as there is a large diversity of fish farming systems. Land based aquaculture facilities require considerable amounts of freshwater which increases the total fresh water demand (see Table 1.1) and usually discharge their wastewater into the surrounding environment, whereas seawater aquaculture facilities produce a series of additional problems such as alteration of water quality parameters on the area (Tovar et al., 2000), modification of benthic ecosystems where the net-cages are located (Riera et al., 2017), interaction and/or pathogen exchange between farmed fish and wildstock (Johansen et al., 2011), especially if the first are genetically modified, and water pollution due to anti-fouling treatments (Mente et al., 2006).

Table 1.1. Water consumption for the rearing of diverse aquaculture species in open systems. Source: Ministry of Agriculture, Fishing and Nutrition, Spain (2002)

Species	***Water requirement (m3/year kg produced)***		
	Minimum	Mean	Maximum
Rainbow trout *(Oncorhynchus mykiss)*	36.9	90.2	220.7
Brown trout (*Salmo trutta*)	52.03	563.5	1075
Turbot *(Scophthalmus maximus)*	157.7	204.9	250.7
Gilthead sea bream *(Sparus aurata)*	83.2	239.7	473.2

Intensification of aquaculture only can lead to the increase of those harmful environmental impacts, therefore the mitigation of those environmental impacts is a matter of particular concern to researchers and global policies. Fish farming system-related environmental impacts probably require more focused research, whereas the reduction of wastes from the aquaculture production is applicable to every fish farming system, making it a very solid starting point to mitigate the integral environmental impact of aquaculture.

Nutrient-rich outputs from fish farms are a consequence of poor understanding and management of the reared fish, likely derived from an inadequate extrapolation of the terrestrial animal farming practices. The aquatic environment makes more difficult to monitor both animal growth and feed utilization than for terrestrial animals (Herath and Satoh, 2015). The lack of precise knowledge of fish biomass often results in overfeeding (Cho and Bureau, 2001). Even if the ration is totally consumed by fish, feeding more protein and phosphorus than required by the fish ultimately leads to the release of excess phosphorus and nitrogen either by incomplete digestion or excretion, and the consequential release on the rearing water (Cho and Bureau, 2001).

Input reduction, particularly fish meal and oil, has also been traditionally very important in academic fields, likely motivated by the stagnant captured fish production. The most promising line of research has been the replacement of fish meal by plant sources to some degree (Martínez-Llorens et al., 2012). Both lines of research must work with one another, because the introduction of alternative diets for fish is another known cause of inefficient feed utilization by fish, leading to additional solid wastes (Davidson et al., 2013).

In any case, as efficient as feed utilization can become, it must be accepted that some waste is going to be produced at some degree, and it is expected to increase with the concomitant world demand. In addition, environmental regulations (*e. g.* Directive 2000/60/EC), concerned with the rising problems of good-quality freshwater availability have been increasing their strictness with wastewater discharge requirements (Martins et al., 2010). Therefore, aquaculturists and researchers must work in waste treatment in addition to waste reduction (Piedrahita, 2003). On the other hand, there are some environmental impacts (escapees, for example) that are site specific, and whilst clean water availability is not certainly a problem for sea-cage aquaculture producing on-growing Atlantic salmon and Mediterranean species, other production systems, species and fish growth stages have been increasingly struggling with availability of water with the very high quality standards required for hatcheries where eggs, larvae and fry are more vulnerable (Blancheton et al., 2013). Water availability and waste treatment issues altogether produce an encouraging interest to move away the production from “natural fish production locations” (ponds, sea cages) to “closed-containment systems”, with optimal management of inputs and outputs. Another additional advantage would be the protection of natural environments.

2. RECIRCULATING AQUACULTURE SYSTEMS

Recirculating aquaculture systems (RAS) are land-based aquaculture farms in which the rearing water is re-used to a certain degree. Recirculating aquaculture systems can be very varied in terms of water inlet and transfer technology, water source and wastewater discharge and treatment (Lekang, 2007). However, they are widely considered intensive aquaculture farms in which production units are manufactured (usually made of fiberglass or concrete) and fish are kept at high densities (Figure 1.2).

RAS were introduced in the early 70s (Liao and Mayo, 1972) as an alternative of traditional systems to reduce the amount of water uptake and to improve quality control. Flow-through aquaculture systems require great quantities of water just to keep certain substances produced by fish (such as nitrogenous (N) and phosphorous (P) substances) at a low concentration within the fish production units or, on the other hand, to keep dissolved oxygen levels within the recommended range for the farmed species. With the development of the aquaculture industry and the scarcity of natural freshwater bodies, water filtration systems and/or water conditioning systems (aeration, heating, etc.) were introduced to reduce the amount of water necessary for pollutant dilution. The introduction of breeding and fry production also motivated the pre-treatment of water inlet to avoid the entrance of potentially dangerous pathogens and unwanted substances, due to the special requirements of early fish growth stages (Murray et al., 2014).

Figure 1.2. Picture of the recirculating aquaculture system (RAS) of the Universitat Politècnica de València.

The re-use of the water drastically reduces the need of water intake and produces a less volumetric effluent discharge (Lekang, 2007). This involves two series of advantages. First, less need of water intake makes the systems are more robust to changes on availability or quality of the source water and the location of the farms can suit specific needs (Singer et al., 2008). Second, the accumulation of pollutants in every recirculation cycle makes water cleaning systems more efficient as they remove more substances per m^3 of water treated (Blancheton et al., 2007). These two advantages make RAS an option to be considered for certain freshwater fish producers who are encouraged to meet certain environmental requirements for discharge water quality. Despite these advantages in environmental impact reduction, recent life cycle assessment of RAS (Aubin et al., 2006; Ayer and Tyedmers, 2009) have pointed out that this fish farming system introduced a new series of environmental impacts, mainly related to increasing global warming potential due to the high energetic requirements (Badiola et al., 2018). Their magnitude is, in global terms, much higher than eutrophication. In that study, however, authors suggested the potential of RAS farms to use energy from renewable sources.

Production in RAS also implies certain advantages from a management view point. Through intensification of water cleaning and recirculation, fish production units can be reduced as much as possible, increasing fish density without altering rearing water quality parameters and therefore rising the profitability of fish/m^2 (Tal et al., 2009). Other notorious advantages are the convenience of fish handling (including harvesting, weighting, grading and medicating (Lekang, 2007)) and water temperature control, maximizing fish yield (Jauralde et al., 2013). Nevertheless, inversion and operational costs are usually much higher than other fish farming systems (Timmons et al., 2009) and water treatment units failure may led to serious complications in fish survival and growth. On top of that, the technological and engineering skills required to correctly run the RAS (Badiola, 2017) may make this rearing system not preferable for most aquaculture companies.

Despite that, the number of RAS facilities have been steadily increasing (Badiola et al., 2012; Martins et al., 2010) and the production of several species on RAS has been developed, mainly freshwater species as rainbow trout (*Oncorhynchus mykiss*), tilapia (*Oreochromis sp*.), or eel (*Anguilla anguilla*). Production of marine species in RAS is not especially relevant as it is difficult to compete with sea-cage farming where high-quality water availability is not a problem. Therefore, it is restricted to high-profitable species

such as turbot (*Scophthalmus maximus*) (Bischoff et al., 2018). The farming of fingerlings of most Mediterranean species is also mostly carried out in RAS (Martins et al., 2010). They are also particularly popular on the Nordic countries, particularly in Norway and the Faeroe Islands (Dalsgaard et al., 2013) thanks to the increasing production of Atlantic Salmon (*Salmo salar*) smolt in RAS. Most new farms prefer RAS as opposed to traditional flow-through systems (FTS) and there is continuous delay on the date of the transfer of smolts to sea cages for the on-growing phase which results in an increasing volumetric production in RAS. The positive results on research conducted comparing smolt performance in flow-through systems and RAS (Kolarevic et al., 2014) have possibly shift the industry in that direction, although for the particular case of the Nordic countries, the unreliability on the quality of their source water and the pumping costs required for procuring it (as most of the source water in the area is groundwater) may make the RAS more attractive than in other zones of Europe (Terjesen et al., 2013). Successful results in rainbow trout rising in RAS, carried out in Europe and USA (Colson et al., 2015; d'orbcastel et al., 2009; Summerfelt et al., 2004) compared to traditional flow-through systems also may increase RAS production.

3. WATER PURIFICATION SYSTEMS IN RECIRCULATING AQUACULTURE SYSTEMS

As previously prefaced, water purification systems are indispensable elements of every RAS and must be designed and operated to meet the water quality standard requirements which may vary with fish species and size (Colt, 2006). The degree of water purification necessity is therefore dependent on the size of the production and the amount of recirculated water (as opposed as new water introduced to the system also called make-up water). As a minimum, RAS must include solid removal systems, dissolved nitrogen removal systems and often water disinfection systems (d'orbcastel et al., 2009; Terjesen et al., 2013), as well as aeration or injection of pure oxygen required by fish and the aerobic bacteria of the biological reactors (Figure 1.3). CO_2 stripping is also recommended, as an excess of gas on the rearing water leads to the apparition of particular diseases, mainly nephrocalcinosis (Colt, 2006). pH management can be done by adding common sodium hydroxide (Timmons et al., 2009), because nitrification performed by the biofilter leads to a continuous acidification of water.

Water purification systems were adopted from wastewater facilities and they are very diverse in technology. Due to wastewater purification being an active field of research,

new equipment and/or improving modifications of existing devices are constantly being developed, produced and gradually implemented on RAS systems all around the world. Kamstra, van der Heul, & Nijhof (1998) reviewed a series of Dutch eel farms with recirculation technologies based on the performance of the ammonia nitrogen removal, which is a fine example of the diversity of water purification technologies found in operating production-scale RAS systems.

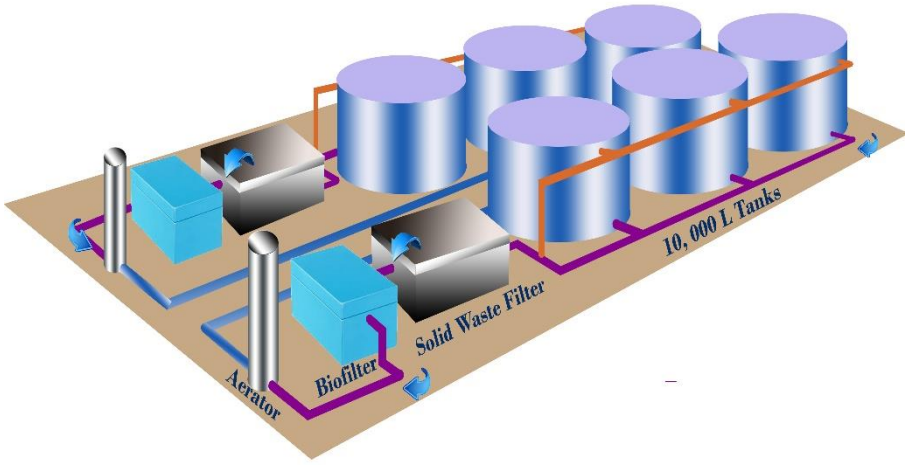

Figure 1.3. Diagram of a recirculating aquaculture system with emphasis on the indispensable water purification units. Source: Aquaflotech, Inc.

The correct way of proceeding while designing the purification systems for a RAS would be: (1) assess the waste production according to farming conditions (fish biomass, tank volumes, quality of feed…), (2) establish a certain water quality criteria and (3) select within the current purification devices the one required to carry out that objective, if possible including slight overestimations of waste production. Studies that perform waste production assessments are continuously being published (Ballestrazzi et al., 1994; Bureau and Hua, 2010; Dosdat et al., 1996; Roque d'Orbcastel et al., 2008), but studies that evaluate the purification efficiency of the new waste removal systems that are day after day being developed, improved and marketed is equally crucial to ensure the correct functioning of recirculating aquaculture systems, because there is a fair risk of overestimating the waste removal potential of a purification device. If let operating performing worse than it is expected to, the accumulation of waste on the rearing water is perfectly plausible as then the reduction of said waste may only possible by discarding portions of water and replacing it with new fresh water (thus reducing the degree of recirculation). Another opposing problem would be to greatly underestimate the waste removal potential and equip purification devices designed to remove much more waste than required by the system, which in itself may impair the correct functioning of the

device and in any case suppose supplementary costs (acquiring the waste removal unit and its operating costs). Efficiency or performance of a purification unit should be stated as % of the target water quality parameter improved/reduced per pass through the unit (Colt et al., 2006) or rate of production/removal of the water quality parameter per unit of time to be adequately integrated as a successful component of a RAS.

Research and development has focused mainly on the understanding and improvement of individual water treatment systems, but additional research is necessary for an integrated approach of the operation of an entire RAS, in which every water treatment units interacts with one another (Badiola et al., 2012; Martins et al., 2010). Furthermore, some researchers have presented several unnoticed problems unresolved with current water treatment equipment, such as the accumulation of other fish and bacteria metabolites (cortisol, for instance) and metals, which can be particularly harmful in fingerling production (Martins et al., 2009).

The present thesis is concerned with ammonia removal units and therefore some other water treatment units will not be discussed in great detail in this section. Solid removal is, however, particularly important because of the influence on the performance of said units. Solids are harmful by themselves. Some studies (Au et al., 2004; Pedersen et al., 2017) indicate that harmful effects of suspended solids are manifested in deterioration of gills, but also there is a direct relationship between the amount of solids and the total microbial activity in the rearing water, mainly opportunistic bacteria that consume oxygen and pose a threat to fish welfare but also compete with nitrifying bacteria for resources and displace them (Michaud et al., 2006), leading to increments on ammonia nitrogen (Davidson et al., 2009) in the RAS. As prefaced above, solids originate from uneaten food (if any) and fish faeces, and they are usually greater the lower digestibility of the diets (Davidson et al., 2013). Many of the papers discussing biofilter performance always recommend a microscreening of the inlet water before entering the trickling filter in particular to reduce the risk of clogging (Crab et al., 2007; Eding et al., 2006; Pedersen et al., 2012). Popular solid removal systems include disc and drum filters (Figure 1.4).

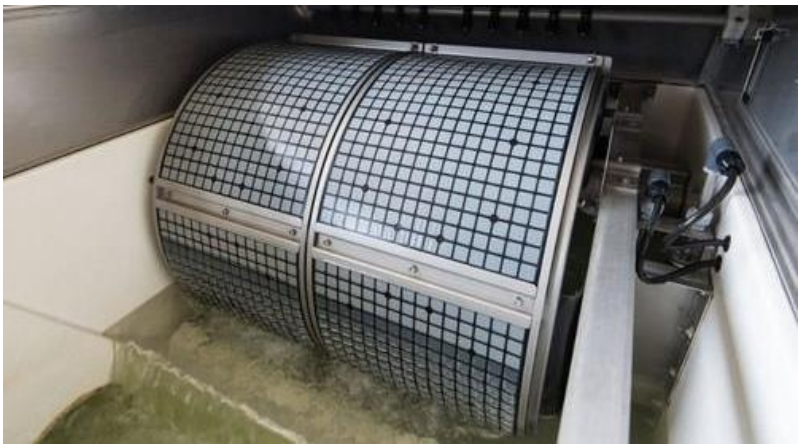

Figure 1.4. Picture of a drum filter, a popular solid removal unit for recirculating aquaculture systems. Source: Global RAS Fishery & Co

4. AMMONIA GENERATION AND TOXICITY

4.1. AMMONIA TOXICITY

This section is dedicated on discussing how and how much ammonia nitrogen is generated by fish, as well as its impact on fish welfare. First of all, ammonia nitrogen is one of the water quality parameters whose monitoring and control in a recirculating aquaculture system is the most critical, aside from dissolved oxygen. There certainly is one of the limiting factors of the water recirculation rate as no removal occurs spontaneously (except their biological transformation due to microbiota present in the surrounding water) and tends to accumulate rapidly with every recirculation cycle. Thus, large quantities of water must be disposed of and replaced with fresh water just to keep the ammonia levels at an acceptable level unless it is eliminated by sophisticated devices that require biology, chemistry and engineering training for understanding and management. In the survey conducted by Badiola et al. (2012), every one of the RAS farms consulted incorporated some ammonia removal unit.

Ammonia is a nitrogenous compound that is produced by fish by deamination of amino acids to produce energy. The high excretion rates in comparison to terrestrial animals are a consequence of the traditionally high protein content in fish diets, adopted due to protein being the major source of energy instead of carbohydrates or fat, the latter of which is more common in terrestrial farm animals (Crab et al., 2007). The protein-rich foodstuff demand is other of the causes on the focus on high-protein retention in reared fish. Ammonia nitrogen is the major component in the nitrogenous excretion of fish,

ranging from 71% to 78% of the total dissolved nitrogen produced (Dalsgaard et al., 2015; Dalsgaard and Pedersen, 2011), although there exist other nitrogenous compounds excreted by fish like urea (around 10%) and other uncharacterized nitrogenous compounds (10%) like proteins or amino acids from the mucus (Kajimura, 2004). Dissolved ammonia exists in two forms, the ionized form (NH_4^+) and the un-ionized form (NH_3). The sum of both forms is denoted total ammonia nitrogen (TAN) and it is usually the term used when discussing waste production on carrying capacity of a recirculating aquaculture systems. The un-ionized form is, however, the most harmful form of ammonia nitrogen and as such it is the parameter that is usually cited by papers stating water quality requirements (Chen et al., 2006; Colt, 2006) or toxicity studies (Dosdat et al., 1996; Lemarié et al., 2004). The ionization equilibrium depends on pH, temperature and salinity (Figure 1.5) and the characterization of those parameters in the rearing water is very important to determine potential toxicity of a given TAN concentration (Tomasso, 1994).

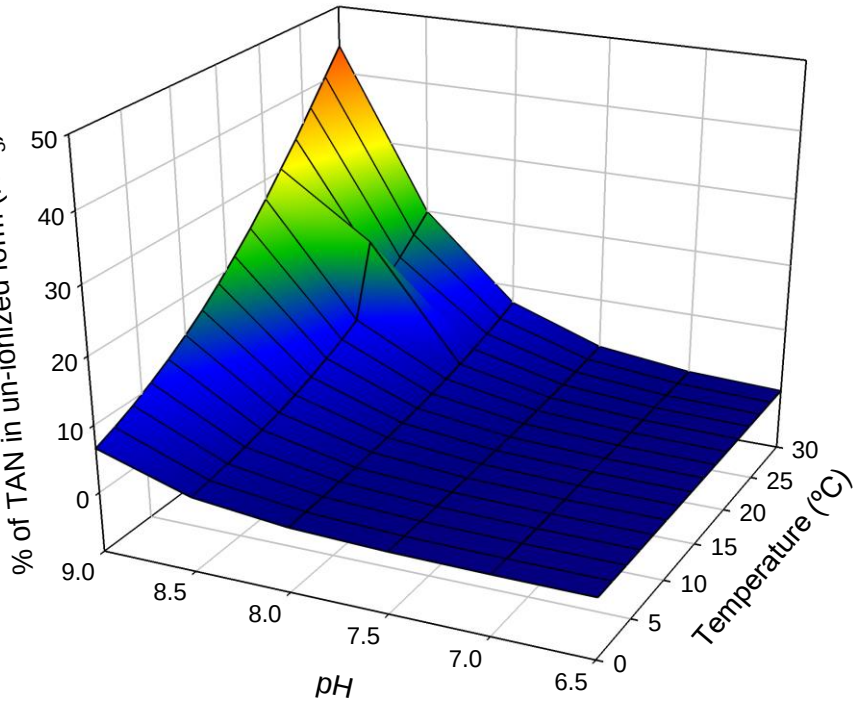

Figure 1.5. % of un-ionized ammonia respect to total ammonia nitrogen depending on temperature and pH in marine water (salinity between 35 and 40 ‰). Source: *Johansson and Wedborg, (1980)*

The higher toxicity of the un-ionized form is relative to its permeability across biological membranes in comparison with the ionized form. Active excretion of ammonia is only possible in its ionized form, so fish are subject to gradients' effect in the uptake or disposal of the un-ionized form. In addition to the toxicity of the un-ionized ammonia, the

constant production of more ammonia within the fish body represents a risk if the surrounding environment does not favor the release of such TAN (Tomasso, 1994). The nature of the toxicity of the un-ionized ammonia nitrogen is further discussed in Tomasso (1994) and Randall & Tsui (2002), but the main effects are considered nervous system malfunctions, gill damage and augmented disease incidence. In practice, high concentrations of this substance may result in death of fish within a few hours if pH and temperature are favorable to the displacement of the equilibrium towards the un-ionized form (Colt, 2006). LC50 (the concentration of toxin that leads to the mortality of the 50% of the study population) and other sub-lethal concentration thresholds (no-observable-effects concentration, lowest-observable effect concentration or reduced growth limit concentration) are useful guidelines to determine toxicity of ammonia nitrogen for a diverse range of species (see Table 1.2). There is no general consensus on recommended minimum concentration as it varies depending on species and life stage, but a range between 0.0125 mg L^{-1} and 0.025 mg L^{-1}, as reported in Chen *et al.* (2006) should be used as a rule of thumb.

Table 1.2. Reduced growth limit concentration of un-ionized ammonia (NH_3) for several aquaculture species.

Species	***Reduced growth limit concentration***	***Reference***
Atlantic cod *(Gadus murhua)*	0.06 mgL^{-1} NH_3	(Foss et al., 2004)
Gilthead sea bream *(Sparus aurata)*	0.27 mgL^{-1} NH_3	(Wajsbrot et al., 1993)
Turbot *(Scophthalmus maximus)*	0.18 mgL^{-1} NH_3	(Person-Le Ruyet et al., 1997)

4.2. AMMONIA EXCRETION

Production of ammonia depends majorly on the feeding intake and protein content of the diet (Dosdat et al., 1996), and a general ammonia production equation can be stated as P_{TAN} = Feeding x Protein content * 0.092 (Timmons et al., 2009), assuming global mean values of protein digestibility and retention. The constant is, in reality, highly variable and depends on several factors besides the quality of the diet, such as the species, growth stage (juveniles show a higher ammonia excretion rates compared to adults), water temperature or protein to energy ratio of the diet. Estimation of waste production for every recirculating aquaculture system is crucial and the literature is very extensive in this

matter. Some results published for common aquaculture species are presented in Table 1.3. Values of expected ammonia production are required for the management of the recirculating aquaculture system and the water purification requirements.

Table 1.3. TAN excretion by diverse aquaculture species. When applicable, the TAN excretion fraction is estimated based on the nitrogenous waste characterization from

Species	TAN excretion	Observations	References
Rainbow trout (*Oncorhynchus mykiss)*	33.8% of ingested nitrogen	Average of three commercial diets: Ecolife 20 from Biomar A/S, 576 BM XS from Aller Aqua A/S and Dan-Ex 2844 from Dana Feed A/S.	(Dalsgaard and Pedersen, 2011)
European sea bass (*Dicentrarchus labrax*)	42.7% of ingested nitrogen	Manufactured diet using fish oil from *Engraulis encrasicolus* as the sole dietary oil source (SIBAL Black Sea Feed Inc., Sinop-Turkey)	(Engin et al., 2013)
Atlantic salmon (*Salmo salar*)	30.75% of ingested nitrogen	Atlantic salmon parr stage. Diet used: Skretting Nutra Olympic 1.2–1.5mm, Skretting, Stavanger, Norway	(Terjesen et al., 2013)
Nile tilapia (*Oreochromis niloticus*)	31.2% of ingested nitrogen	Commercial diet supplied by Tongyi Co., Ltd., Guangdong, China. 33% crude protein. Estimation from total dissolved nitrogen recovered in the study	(Cao et al., 2019)

5. AMMONIA REMOVAL

Although physical-chemical removal of ammonia is technically possible, the most common ammonia removing techniques in RAS imply some degree of biological action. Either large quantities of "waste water" (*i.e.* water with a high TAN concentration) are replaced with clean water or make-up water and the wastewater is carried to dedicated treatment ponds whose purpose is the stabilization of the waste and transformation in other valuable sub-products (Crab et al., 2007) or the rearing water is directly derived to treatment units containing bacteria transforming TAN to less toxic substances. Although treatment ponds and other similar ways of nutrient removal (bioflocs, for instance) are relatively inexpensive and less prone to failure, they require extensive surfaces that are often impractical for intensive aquaculture. Biofiltration in dedicated ammonia removal units are, in essence, the most common technology applied in modern RAS (Timmons et al., 2009). Although some nitrogen is produced in solid form and therefore can be removed with solid removal technologies, this fraction is small in the total waste production (6.8 % of ingested nitrogen in rainbow trout *(Oncorhynchus mykiss*) as reported in Dalsgaard and Pedersen, 2011). Thus, the vast majority of nitrogen removal units involve the uptake of dissolved nitrogen (ammonia) from water and the release of more oxidized forms.

5.1. NITRIFICATION

The transformation of ammonia nitrogen to nitrate is called nitrification and is a two-step reaction carried out by two phylogenetically different types of bacteria in succession (Hagopian and Riley, 1998). The first step, also called nitrition, consists in the catabolization of the ammonium ion (NH_4^+) to nitrite while the second step, also called nitration, consists in the mineralization of nitrite to nitrate. The first step is performed by ammonia oxidizing bacteria (bacteria of the genera *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio*) and the second step is performed by nitrite oxidizing bacteria (bacteria of the genera *Nitrobacter*, *Nitrococcus*, *Nitrospira*, and *Nitrospina*) (Sakami et al., 2012). The intermediate product, nitrite, is also toxic to fish, causing methemoglobinemia (more commonly referred to as brown-blood disease), due to the oxidation of iron in the hemoglobin molecule (Tomasso, 1994) but it is usually transformed to nitrate as soon as it is produced, provided both colonies of bacteria are present in the biofilter (Timmons et al., 2009). The biochemical reactions that conform nitrification are as follows (Eding et al., 2006):

Nitrition: 80.7 NH_4^+ + 114.55 O_2 + 160.4 HCO_3^- → $C_5H_7NO_2$ + 79.7 NO_2^- + 82.7H_2O + 155.4 H_2CO_3

Nitration:134 NO_2^- + NH_4^+ + 62.25 O_2 + HCO_3^- + 4H_2CO_3 → $C_5H_7NO_2$ + 134.5 NO_3- + 3 H_2O

O_2 and HCO_3^- are also indispensable for the chemical reactions to take place. O_2 as the nitrifying bacteria are strict aerobes and alkalinity as the source of carbon for biofilm mass. The final products are water, nitrate and $C_5H_7NO_2$ which represents the cell biomass growth. The oxygen demand is quite high and in poor aerated biofilms it can become a limiting factor on the ammonia removal. Nitrifiers are able to survive with this conditions until oxygen is once again available (Hagopian and Riley, 1998) but biofilter performance would be affected. Due to the consumption of alkalinity a slight decrease in pH can be observed over time, therefore it should be corrected, for example, by adding sodium bicarbonate in the water. Nitrifiers are prone to adhere themselves and form biofilms using extracellular polymers rather than remaining in the water column in free form which has been taken advantage of by using solid carriers of different shapes and forms have been used to stimulate the aggregation of a thick biofilm to use as a biochemical reaction unit.

5.2. NITRIFYING FILTERS CLASSIFICATION

There exists a large variety of biofilters that are used in RAS nowadays, with different strengths and weaknesses. Considerations for biofilter design include, as mentioned earlier, the possibility of oxygenation for the nitrification reactions, the stability of the biofilm (including proper expansion of the beds when applicable), the prevention of head loss which requires more pump power and energy and, on the other hand, reduced cost and high performance.

Biofilters can be differentiated under several criteria, including if they are suspended growth-based or fixed film-based, emerged or submerged, or by the biofilm management technique. Malone and Pfeiffer (2006) reported a very complete classification of the existing types of nitrifying filters at the time. Suspended growth systems leave the biofilm free in the water column and are particularly vulnerable to the washout of nitrifying

bacteria (Timmons et al., 2009). Fixed film filters use solid media surfaces for the bacteria to attach and grow, which may be composed of rocks, sand, plastic or ceramic materials.

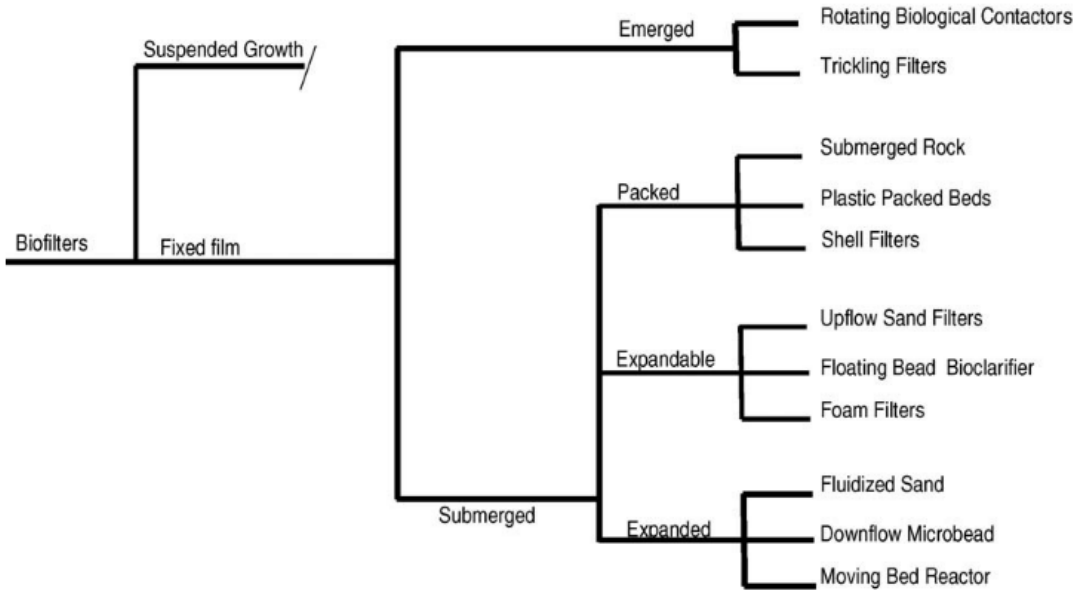

Figure 1.6. Classification of nitrifying biofilters based on biofilm characteristics. Source: *Malone and Pfeiffer, (2006)*

In fixed film filters, however, heterotrophic bacteria can also be attached to the solid surface and, as their specific growth rate is an order of magnitude higher than nitrifiers (Hagopian and Riley, 1998), they can easily outperform them and therefore a loss of performance can occur if no actions to manage biofilm growth are taken. An excessive biofilm growth would isolate the nitrifying bacteria to the inner layers of the biofilm while the outer layers would be colonized by heterotrophs which would limit the diffusion rate of TAN and would consume the oxygen faster than the autotrophs (Blancheton et al., 2013). As such, biofilm management is a key element in the design of biofilters.

On the other hand, biofilms can be emerged or submerged. The principal advantage of emerged biofilters is the ability of procuring oxygen for the nitrifying bacteria as opposed to submerged biofilters, and can use biofilm sloughing as a means for biofilm management by the impact between water and the biofilm through several methods. The main disadvantages are usually the risk of clogging from the sloughed biofilm (Timmons et al., 2009) and the head loss as they usually are to be placed elevated from the fish tank effluents. There are two types of emerged biofilters, one of which (trickling filter) will be the object of this thesis. The other type, the rotating biological contractor, is another popular type of biofilter in aquaculture whose main trait is that the filter media surface is

set in a circular motion over a longitudinal axis, and the biofilm alternates between water and air, being always wet but with the possibility of taking oxygen directly from the surrounding air. This is particularly more advantageous from the head loss perspective as the flow is horizontal and it does not require the lifting of the water in an upwards or downwards configuration (Brazil, 2006).

Submerged biofilters require additional management for oxygen transport to the biofilter area to ensure nitrification (via low hydraulic retention times or aeration), but no additional structures are required to conduct the water to the treatment units and therefore biofilters can be very voluminous with generally little drawbacks, including head loss. The media may be static, expanded (media is always in motion within the vessel) or in an intermediate category called “expandable”, which is a static media bed that is set in motion on occasion to produce abrasion and remove the outer layers of an excessively large biofilm (Malone and Pfeiffer, 2006). Filter media of those biofilters present generally higher specific surface media than the filter media of emerged filters. Microbeads with a diameter as small as 1 mm (3936 m^2/m^3) have been used as biofilm carriers (Greiner and Timmons, 1998).

5.3. TRICKLING FILTERS

Trickling filters (Figure 1.7) are a particular type of fixed film biofilters in which water trickles down through a packed filter media substrate in which the biofilm is attached. Water must flow downwards the filter and therefore some head loss is inevitable in order to carry the influent to the top of the filter. Their main advantages are their simplicity, availability of oxygen and possibility of CO_2 stripping and good performance in general (Crab et al., 2007).

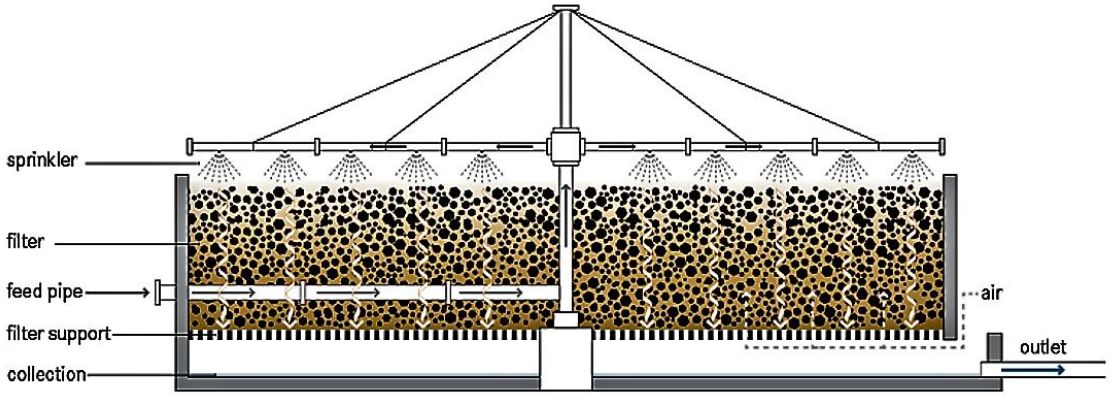

Figure 1.7. Schematic drawing of a trickling filter. Source: Tilley *et al.*, (2008)

Trickling filters take advantage of high hydraulic loading rates (water flow per cross-sectional area of the biofilter, established as m^3/m^2 h), which grant the vessel the behavior of a plug-flow reactor which in turn is beneficial to improve nitrification rates, based on the accumulation of the heterotrophs in the top layers and the nitrifiers in the middle and bottom layers (Watten and Sibrell, 2006). Some researchers have stated positive influences on higher hydraulic loadings on the ammonia removal rates (Kamstra et al., 1998; Nijhof, 1995). Different types of media (Figure 1.8) have been used as a constituent of the biofilter, generally designed with the objective of augmenting the specific surface area and/or void ratio. Specific surface area of the media currently available is not, however, as high as the media used for submerged biofilters, varying between 100 and 300 m^2/m^3 (Lekang and Kleppe, 2000). Despite not being the most technologically advanced their simplicity makes it a very popular nitrifying filter for new and existing RAS systems (Kamstra et al., 1998), and there is still room from improvement based on the introduction of new filter media.

Figure 1.8. Examples of filter media found in common trickling filters.

There are still relatively few studies that analyze biofilter performance of RAS for rearing marine species (Kumar et al., 2011; Nijhof and Bovendeur, 1990). It is still debated whether a certain salinity is detrimental to the nitrification capability (Malone and Pfeiffer, 2006), although it is known that the maturation period of the biofilm to achieve its full-grown phase is larger (Nijhof and Bovendeur, 1990). Therefore, procuring data on nitrification rates for saltwater systems is a step necessary to gradually introduce the culture of marine species on RAS instead of sea cages and trickling filters are a good candidate for ammonia removal under these circumstances as the supply of oxygen is not a problem, there is no need for additional carbon dioxide stripping and the sizing of trickling filters depending on estimated waste production for a new established farm is simple.

Performance of trickling filters (measured as ammonia eliminated by surface or volume and time) is largely dependent on the filter media used. As there is a considerable dependence respect to the TAN concentration of the inlet, rates are usually reported at a given concentration or include some additional factors that consider it. Kamstra et al. (1998) reported nitrification rates between 0.24 and 0.55 g/m^2 day with feed loads between 1.60 and 2.92 kg/m^3 from commercial farms using Bionet®, Filterpak® and Munters® filter media. Greiner and Timmons (1998), on laboratory conditions at much higher hydraulic loadings, reported rates from 0.94 to 3.92 g/m^2 day, using Norpak® filter media, for influent TAN concentrations between 0.81 and 4.63 mg/L.

5.4. FACTORS AFFECTING NITRIFICATION PERFORMANCE

The conversion of ammonia to nitrate is a live process and, therefore, parameters affecting nitrifiers' survival and growth ultimately determine the amount of ammonia removed from water, if any, at a given time. At the same time, like any chemical reaction, its kinetics are determined by several factors that must be understood in order to obtain

the maximum rate of nitrification possible. Even if two biofilters are identical in biofiltration area, certain biotic and abiotic factors may lead to the better performance of one biofilter over another (Chen et al., 2006; Eding et al., 2006). Some of this factors are "reactor-specific" meaning that the value of a certain parameter (hydraulic loading for instance) may be beneficial for trickling filters but no so much to suspended growth filters (Malone and Pfeiffer, 2006) and other factors may affect all nitrification systems by a similar degree. A classic approach to estimate the influence of a certain parameter on nitrification is to experimentally evaluate how it increases or decreases the nitrification rate or ammonia removal rate (gTAN eliminated/time), and it will be used throughout the present thesis as well.

One of the factors that has been more extensively studied is the substrate concentration influence on nitrification rates or, more simply put, nitrification kinetics (Bovendeur et al., 1987; Greiner and Timmons, 1998; Zhu and Chen, 1999). The most common consensus is that the nitrification reaction is substrate-dependent but the half saturation constants reported have slightly varied over time (Watten and Sibrell, 2006). This is applicable for both reactions. The maximum TAN concentration in which the reactions become independent of substrate for trickling filters has been reported as 2.5 mg L^{-1} TAN (Greiner and Timmons, 1998), with no oxygen diffusion limitations. It is suggested to operate a biofilter below this threshold as a safety measure to prevent accumulation of ammonia in the rearing water in case of a sudden increase (Eding et al., 2006).

Other of the most important factors that affects nitrification rates in the presence of heterotrophic bacteria. As the colonization of the biofiler by them is largely a consequence of organic carbon from faecal waste, several papers have used the C/N parameter, or analogously the COD or BOD concentration of the influents of nitrifying filters as a measure to conduct experiments about the effects of heterotrophic bacteria on nitrification. Thus, Zhu & Chen (2001) demonstrated a reduction of nitrification performance (expressed as TAN concertation alongside a series reactor) with increasing values of C/N ratios. A similar study (Ling and Chen, 2005) was carried out in different biofilters, a floating bead filter, a fluidized sand filter, and a submerged bio-cube filter. The reduction of nitrification rates was about 70% when the C/N ratio increased from 0 to 3. The same authors also converted experimental conditions (C/N ratios) intro COD/N for clarification and comparison with other studies, which used COD as an indicator for heterotrophic bacteria disturbance in a nitrifying trickling filters. Technically speaking,

BOD would be a better indicator (Malone and Pfeiffer, 2006), but COD has been accepted as an appropriate measure.

Several studies have been carried out about the influence of temperature on nitrification (Salvetti et al., 2006; Zhang et al., 2014; Zhu and Chen, 2002) which conclude that the effect of temperature on the nitrification reaction is less than predicted by the general Arrhenius equation. Provided that the temperature is appropriate to nitrifying bacteria (5ºC-30ºC), there are no inhibitions of nitrification to be expected. If anything, it is discussed if oxygen diffusion changes could be responsible of masking temperature effects (Okey and Albertson, 1989). Other parameters such as pH, alkalinity, salinity, nitrite and nitrate can also affect nitrification, although their effects are less significant. pH may be responsible for nitrification rate drops as affecting nitrifying bacteria which have an optimum pH for survival (pH 6–9 for *Nitrosomonas* and 6.3-9.4 for *Nitrobacter* (Timmons et al., 2009)).

In the particular case of the trickling filters, there are other process parameters that affect nitrification rates. The first one is the hydraulic loading, whose improvements on ammonia removal can be explained by improved wetting (Eding et al., 2006), confinement of the heterotrophic bacteria on the top layers and favoring of the formation of a nitrifying biofilm across a greater part of the vessel (Nijhof, 1995). Nevertheless, it must be considered that there probably is a limit on the benefits on the overall performance of the RAS in general. On the one hand, some studies, like the paper of Greiner and Timmons (1998) have already suggested that there is a limit on the improvement of nitrification by increasing hydraulic loading alone but, on the other hand, either if the hydraulic loading is increased by increasing water flow over all or reducing the biofilter diameter, it will suppose an increment of the power of pumping necessary. A simple solution to reduce head loss (compared to a bigger biofilter) is to establish several biofilters in parallel before returning the water to the system, as carried out in Greiner and Timmons (1998).

The second process parameter important to the performance of trickling filters are the characteristics of biofilter media. Traditionally in biofilter engineering the specific surface area (available surface for nitrification/volume) has been considered the basis of determining biofilter media quality (Malone and Pfeiffer, 2006), although some other factors like porosity, void ratio and ability to retain the nitrifying biofilm may be some differentiating factors as well. The is a considerable myriad of filter medias out there,

made from sponges, rocks, grass or plastic. Both the nitrifying rates and the hydraulic loading limits (both superior and inferior) are dependent on filter media, therefore the modeling of the biofilters with undocumented filter media is always tricky as ammonia removal rates may be different than expected.

JUSTIFICATION AND OBJECTIVES

Trickling filters are a very relevant candidate to serve as ammonia removal units for new recirculating aquaculture systems, as they are easy to operate, design and build, they do not require additional oxygenation and serve as a CO_2 degassing unit as well. Their performance is relatively high and the media is inexpensive. However, as any biochemical reactor, there are numerous factors that influence on their performance that must be studied, determined and quantified to be able to predict their capacity to remove the TAN required for the correct operation of a recirculating aquaculture system.

The determination of the effect of single process parameters have been well documented (Greiner and Timmons, 1998; Kamstra et al., 1998; Zhu and Chen, 2002) but reports of the interaction of process parameters with each other and their effects on the overall ammonia removal rates are lacking. Furthermore, research is usually carried out in freshwater considering species such as rainbow trout (*Oncorhynchus mykiss*) or European eel (*Anguilla anguilla*), and studies carried out in marine water are scarcer, despite the suitability of recirculating aquaculture systems for the rearing of species such as turbot (*Scophthalmus maximus*) due to their tolerance to high densities. The rearing of other marine species usually reared in sea cages would also be convenient in RAS, as this would reduce the environmental impact of their production and would compensate the deleterious effect of the low sea temperatures in winter on fish growth. Finally, there are no studies as well that determine ammonia concentration with biofilter in operation. Consequently, high ammonia removal rates obtained as a result of high ammonia loads can conceal negative effects that the results of this thesis bring to light, such as the existence of peaks of high ammonia concentration.

Studies performed with live fish are also infrequent. The measurement of ammonia excretion based on several factors is present in several papers (Ballestrazzi et al., 1994; Dosdat et al., 1996; Echevarría et al., 1993; Estruch et al., 2018) but there is no literature about the integration of the nitrogenous waste estimation and the nitrogenous waste removal determination, which would be convenient to offer a more holistic approach to biofilter performance in recirculating aquaculture systems. Moreover, studies of ammonia removal with live fish would be interesting to fully determine the importance of the influence of the organic matter on nitrification impairment.

The present thesis serves as a contribution of the general understanding of the effect of the combination of process parameters on mean ammonia removal rates and the ammonia concentrations that are present on the tank water during operation as a result

of such performance. The estimation of biofilter performance (ammonia removal rate and maximum N-TAN concentrations) is also carried out with live fish and the ammonia generated is dependent on fish excretion, which in turn depends on feed characteristics.

The specific objectives were:

- The determination of biofilter performance (ammonia removal rates and maximum N-TAN concentrations) depending on filter media type, temperature and ammonia load, as well as their interaction

- The determination of biofilter performance (ammonia removal rates and maximum N-TAN concentrations) depending on hydraulic loading, temperature and ammonia load, as well as their interaction, using as biofilm carriers the filter media which yielded the best results in the previous experiment.

- The rearing of a group of gilthead sea bream (*Sparus aurata*) by using the biofilter configuration that granted the best performance among all the different combination of process parameters, using different diets and feeding strategies, to determine the viability of the pilot recirculating aquaculture systems, as well as the determination of ammonia excretion and oxygen consumption depending on diet and feeding strategy.

- The determination of biofilter performance (ammonia removal rates and maximum N-TAN concentrations) depending on diet and feeding strategy of the fish reared in the system, with emphasis on the relationship between the ammonia excreted by the fish (and therefore ammonia load on the influent) and the ammonia removed by the biofilter.

SUMMARY OF EXPERIMENTS

1. Determination of N-TAN variation and mean N-TAN removal rate (n=6) depending on the combination of three process parameters (*filter media, temperature and ammonia load*), with three specific values each, for a total of 27 unique combinations.

2. Determination of N-TAN variation and mean N-TAN removal rate (n=6) depending on the combination of three process parameters (*hydraulic loading, temperature and ammonia load*), using Bactoballs® as filter media, with three hydraulic loading and ammonia load values as well as five temperature values, for a total of 45 unique combinations.

3. Determination of juvenile gilthead sea bream (*Sparus aurata*) ammonia excretion and oxygen consumption, as well as N-TAN variation and mean N-TAN removal rate (n=6) of the biofilters depending on the combination of diet and feeding strategy used to feed the fish during a rearing trial, using three different diets and three different feeding strategies with notably different nutritional values.

DECLARATION OF INTERESTS

The research articles that compose the entirety of this thesis have been supported by the national project “Design of a recirculating aquaculture system for aquaculture plants (2011–2014)” funded by Ministry of Science and Innovation, Spain; as well as partially by the project “Design of a recirculating system”, by Universitat Politècnica de Valencia. The authors declare no conflict of interests and willingly accept the inclusion of the contributions in the present PhD thesis.

MATERIAL AND METHODS

In this particular thesis there has been a variety of chemical analytical methods, with several ways to analyze certain components that have been improved and varied over time in terms of speed, commodity and precision. At the same time, there has been some alterations in the experimental system design and operation alongside the three main complete experiments that compose Chapters 3, 4 and 5. For this very reason, it does not seem reasonable to develop a continuous material and methods section applicable to every contribution and the material and methods section of every one of the published or in ways of publication research articles will be left untouched as a manner to describe which were the precise experimental designs, research units, chemical analysis and statistics performed for every different trial. In spite of that, this section will include and document the general specifications of the facilities common to every experiment, a general description of every piece of equipment used on the research as well as some bullet points on the chemical analysis employed and the calculations performed.

1. Facilities

Every experiment included in this thesis was performed in the Laboratory of Aquaculture, facility of the Universitat Politècnica de Valencia Figure 2.1. The main experimental system consisted of a series of six individual sub-systems (Figure 2.2) with no connection to each other. Every single one consisted in a rearing tank (fiberglass cylindrical tanks with 750 L of maximum capacity), a biofilter with different volumes depending on the experiment, and a small pump (Oceanrunner® OR3500, Aqua-Medic®, Bissendorf, Germany) to flow the water from the outlet of the tank to the biofilter (Figure 2.3).

Figure 2.1. Picture of the experimental system

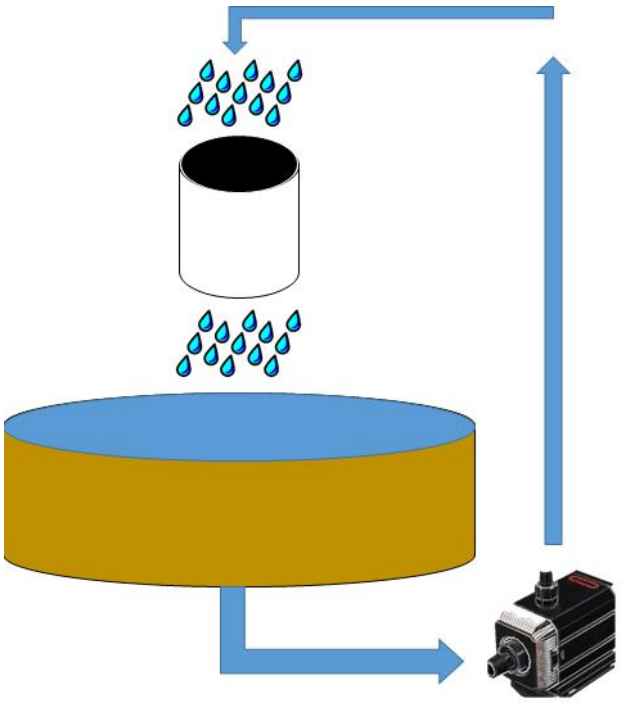

Figure 2.2. Diagram of each one of the six individual systems where all the experiments on this thesis took place.

Figure 2.3. Detail of the pump used to recirculate the water

Figure 2.4. Detail of the inlet of water to the biofilter

Biofilters, for the most part, were home-made consisting on buckets or covered plastic nets containing the filter media and thus the biofilm (Figure 2.4). The filter volume was determined by the volume of filter media (π x r^2 x height), and volume was modified by adding or subtracting filter media from the container. The container was also drilled by the bottom, allowing the water to be released from the biofilter, and return to the tank. Water flow, if needed, could be regulated by the strangulation of the pump, partially closing one of the valves equipped by this very reason. On occasion some additional elements were added to the systems, like internal heaters to control water temperature, or a solid removal unit. Those additional systems will be described in the corresponding chapters.

Different filter media were used to fill the vessels for each of the three different experiments. The first set of trials (Chapter 3) tested three different filter media, which are shown in Figure 2.5. For the later trials (Chapter 4 and 5) biofilters were composed of the filter media Bactoballs® that proved to be the most convenient in Chapter 3.

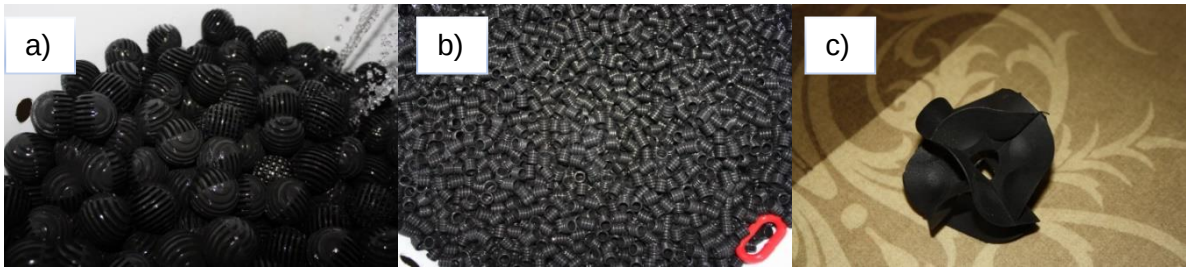

Figure 2.5. Filter media used in the present thesis: a) Bactoballs® b) MECHpro® rings c) Type A Biofill

The tanks were filled with marine water. Except on the case of the experiment carried out with live fish, water was manufactured using dechlorinated tap water and adding the corresponding amount of sea salt (Sea salt for human consumption, Salinera Española S.A., San Pedro del Pinatar, Murcia, Spain). Water used for the rearing of gilthead sea bream (*Sparus aurata*) was brought from the nearby Mediterranean Sea with a tank truck. When water was to be remaining on the tank for a relatively long period (for example when starting up the biofilter, in the period between experimentations or when using alive fish), water salinity was routinely checked with a commercial refractometer and, if necessary, it was adjusted by adding freshwater (dechlorinated tap water).

2. Analytical protocols and equipment

The main determinations performed in every trials are the TAN concentration, pH, dissolved oxygen, temperature, and occasionally nitrite, nitrate and alkalinity. Samples were taken from the tank water, or were performed directly inside the tank in the case of dissolved oxygen and temperature, using portable equipment. pH was measured on a Thermo Scientific™ Orion™ 4-Star Plus pH/ISE Benchtop Multiparameter meter using the pH electrode for most of the experiments, and dissolved oxygen and temperature was measured using a Handy Polaris® oximeter (OxyGuard®, Farum, Denmark).

TAN, nitrite, nitrate and alkalinity were determined by spectrophotometry. Several equipment was used depending on the chemical species analyzed, the experiment and the protocol used for determination. Most of the analyses were carried out in a stationary laboratory T60V UV-VIS spectrophotometer (PG Instruments, Leicester, UK) (Figure 2.6), some of the chemical determinations were carried out in a microplate reader (Victor 1420 microplate reader, Perkin Elmer, formerly Wallac Oy, Massachussets, USA) (Figure 2.7). Alkalinity, on the other hand, was measured in a portable “checker” (Hanna Instruments®, USA), specially designed for marine water.

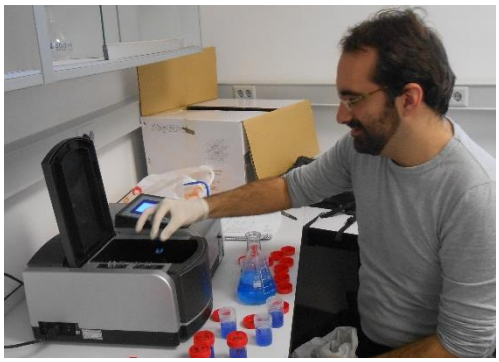

Figure 2.6. Determination of TAN (total ammonia nitrogen, including both the ionized and the un-ionized form) with the T60UV-VIS Spectrophotometer, using the indo-phenol method.

Figure 2.7. Picture of the microplate reader used for nitrate determination.

Samples were taken from the outlet of the biofilter (carefully making sure that water flowing from several holes was grabbed) and carried to the laboratory area of the main building. pH was immediately measured, while TAN, nitrite or nitrate determinations, when not rapidly possible, were performed the following days and the sample was placed on a refrigerator until analysis. If analyses were postponed for several days or weeks, the samples were frozen at -18ºC, after filtering the samples.

The third experiment (Chapter 5) was performed with live fish and manufactured diets Gilthead sea bream (*Sparus aurata*) as well as the control diet were obtained from a local fry producing company. Diets (excluding the control diet) were made in the feed producing laboratory of the Universitat Politècnica de València. The ingredients were mixed and extruded with a semi-industrial twin-screw extruder (CLEXTRAL® BC-45, St. Etienne, France) (Figure 2.8). The processing conditions were: 0.63 g screw speed, 110 °C and 30–40 atm.

For this third experiment additional analysis involving nutritional aspects such as protein or fat content were involved. To that extent, dry matter and ash content of fish and diets were also analyzed. Dry matter was analyzed by drying the samples in a heat oven at 105 ºC to constant weight. Ash content was determined by incinerating the samples in a muffle furnace at 550 ºC for 6 hours. Protein content was determined by the Dumas principle in a LECO determinator (Leco Corporation, St. Joseph, USA) and fat by extraction with dimethyl-ether (ANKOMXT10 Extractor).

Figure 2.8. Picture of the semi-industrial extruder used to manufacture the experimental diets used in the study in the feed producing laboratory of the Universitat Politècnica de València.

Every experiment had in common that the nitrogenous species (ammonia, and nitrite and nitrate when applicable) were measured every two hours to detect the post-prandial ammonia peaks (even though feeding periods were simulated in Chapter 3 and 4) and therefore the maximum N-TAN concentration. Moreover, the continuous N-TAN measurement allowed for the identification of the point of total ammonia removal, if it took place, as well as the determination of if the biofilter was capable of removing the total of the ammonia produced or added before a new day, detecting possible accumulations of ammonia over time. Thus, the daily ammonia removal rates alongside this thesis are defined as the amount of ammonia added/produced minus the remaining ammonia after 24 hours divided by biofiltration area.

CHAPTER 3

Influence of filter medium type, temperature and ammonia production on nitrifying trickling filters performance

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ABSTRACT

This work focuses on the achieving of optimal design and modelling of nitrifying trickling filters for closed circuit aquaculture turbot *(Scophthalmus maximus)* farms. Several process parameters influential in nitrifying filtration were established on experimental biofilters and their efficiency was tested, based on the removal of nitrogen contained in total ammonia nitrogen (N-TAN) in a fixed time (24 hours). Those process parameters were filter media types (Type A Biofill®, Bactoballs® and MECHpro® rings), temperatures (24.3ºC, 19.0ºC, 15.3ºC) and TAN loads (3, 6 and 9 g per cubic meter per day) while other process parameters values remained constant. TAN production was simulated with the addition of ammonium chloride (NH_4Cl) in the recirculation system. Constant measuring of the total ammonia nitrogen concentration in the biofilter effluent was required to perform a model of N-TAN fluctuation based on a specific feeding regime and to ascertain performance differences between biofilters.

At the end of the experiment, notable differences were observed in the ammonia removal rates depending on different process parameters. The MECHpro® filter medium led to the lowest mean N-TAN removal rates (0.078 g N-TAN removed m^{-2} day^{-1}). The N-TAN removal rate generally increased with higher temperatures, the trials with the highest mean temperature (24.3ºC) led to the highest mean N-TAN removal rate (0.26 g N-TAN removed m^{-2} day^{-1}). Similarly, the N-TAN removal rate increased with high TAN load. The trials in which the TAN load was 4.5 g per day showed the highest N-TAN mean removal rate (0.27 g N-TAN removed m^{-2} day^{-1}).

1. Introduction

Aquaculture farms in land in which the culture water is recycled are gradually increasing during the course of the current century (Murray et al., 2014). In those farms, water is constantly moved by pumps in closed circuits called recirculating aquaculture systems (RAS). Recirculation of water in aquaculture presents an important alternative to traditional production methods, because it means independency of natural water resources and allows manipulating water characteristics (Guerdat et al., 2013), the temperature being the most important, because fish have an optimal temperature on which their growth is remarkably better, as observed for different species, such as gilthead sea bream *(Sparus aurata)* (Lupatsch et al., 1998), European sea bass *(Dicentrarchus labrax)* (Lupatsch et al., 2001), and white grouper *(Epinephelus aeneus)*

(Lupatsch and Kissil, 2005). Another important feature of the use of recycled water for in-land aquaculture farms is that production volume can be increased by height without need surface increase (Martins et al., 2010). Turbot *(Scophthalmus maximus)* is an excellent candidate for production in those systems due to its benthic nature and the capability of living under relatively high stocking densities (Irwin et al., 1999). Those characteristics properly allow to culture turbot in tanks with low water volume. In fact, stocking densities in commercial turbot aquaculture, cultured in tanks or in sea water cages, have reached 25 to 30 kg m^{-3} (Irwin et al., 1999). It is also a fast-growing aquatic species of great economic value (Foss et al., 2007).

A well designed RAS allows keeping water quality optimal, because all parameters are controllable and manageable by the producer. Parameters that are influential with respect to the correct operation of an RAS are oxygen (O_2) and carbon dioxide (CO_2), organic matter, pH, suspended solids (SS), alkalinity, hardness, nitrite, nitrate or total ammonia nitrogen (TAN), plus the presence of opportunistic pathogens, as they determine the survival and optimal growth of the fish (Portz et al., 2006). All the above mentioned water quality parameters unavoidably deteriorate with every recirculation cycle. Oxygen is rapidly consumed by the fish and, depending of the feed intake and the feed properties, organic matter (both dissolved and suspended) and ammonia are produced and accumulated in the bulk water. The latter is the most threatening product (Hargreaves, 1998) and the increment of a concentration beyond certain limits can be really detrimental for fish health (Eddy, 2005) as it lowers the ammonia excretion and produces an accumulation of ammonia levels in blood, causing nervous system problems and death (Wilkie, 1997).

Fixed-film biological filters are usually installed in aquaculture facilities with the main goal of TAN removal (Eding et al., 2006). They consist in a series of diverse solid surfaces where a population of nitrifying bacteria attaches and grows with excreted extra-cellular polymers (Hagopian and Riley, 1998). Four kinds of biofilters are commonly used: rotating biological contactors, trickling filters, bead filters and fluidised sand biofilters (Crab et al., 2007). Among the other possibilities, trickling filters display relatively high removal rates (Greiner and Timmons, 1998). Besides, they have considerable advantages: low price, the oxygen transfer is provided as water cascades directly over the media (Malone and Pfeiffer, 2006) and degasification of CO_2 and simplicity of design, construction, operation and management (Eding et al., 2006). Because of that, the trickling filter was used for this study.

TAN load is directly correlated to the fish production plan of aquaculture facilities and usually determines the volume of biofilters installed and the pumping requirements. Nevertheless, a wide range of process parameters apart from biofilter size affects the speed of TAN removal (and therefore the achievement of the targets in TAN concentration), meaning that the proper design and evaluation of performance is essential to avoid the construction of larger trickling filters than needed and the related expenses. A large number of process parameters affects the nitrification rate, for example influent concentration of TAN and oxygen, organic matter, nitrite, temperature, alkalinity, pH and hydraulic loading (Eding et al., 2006). Although models have been constructed to approximate the influence of process parameters of the biofilm on TAN removal efficiency by applying the nitrification kinetics theory (Bovendeur et al., 1987; Nijhof, 1995; Watten and Sibrell, 2006), the influence of several process parameters on the rates at which nitrification reactions take place still have to be assessed empirically. For example, Nijhof (Nijhof, 1995) included in his model “a” and “b” parameters depending on external factors, or internal proprieties, and presented an equation relating the value of “a” with several hydraulic loadings. Kamstra, van der Heul and Nijhof, (1998), validated Nijhof’s model and observed several variations of predicted TAN removal rates depending on the filter medium type.

Three of these process parameters were tested in this article. One of them is the TAN load, which acts as a limiting substrate. Kinetics of the nitrification reaction are described by the Monod-type expression (Rittmann and McCarty, 1980; Srna and Baggaley, 1975; van Rijn, 1996). Besides, experimental procedures that demonstrate a relation between the TAN concentration in the influent and biofilter performance (Greiner and Timmons, 1998; Kamstra et al., 1998; van Rijn and Rivera, 1990; Zhu and Chen, 1999) have also been performed. Yet Bovendeur, Eding and Henken, (1987), noticed that the nitrification reaction is sometimes independent of the substrate when oxygen acts a limiting factor, which is not desirable for the culture. Greiner and Timmons (Greiner and Timmons, 1998) also reported 0-order reactions in their research at high TAN concentrations (above 2.5 mg L^{-1}). Nonetheless, these articles often analyze the impact of a steady TAN concentration rather than analyze the effect of a fluctuating TAN concentration produced by the shifting excretion rate during the day occurring after the feeding, observed in turbot (Dosdat et al., 1996) as well as in other teleost species (García-Romero et al., 2014). In the present research, three TAN loads (defined as the total amount of TAN produced during a day in a standard aquaculture farm) are tested, and the N-TAN concentration is monitored during 24 hours.

The filter medium type was another of the process parameters tested in this study. The influence of different filter media types on the TAN removal rate of trickling filters has been studied in several articles (Kamstra et al., 1998; Lekang and Kleppe, 2000), although the influence of the filter medium type is often discussed when analyzing the performance of all kind of biofilters (Crab et al., 2007; Greiner and Timmons, 1998; Timmons et al., 2006)**.** Characteristics of the filter media types considered to affect the performance of the nitrifying trickling filter include void ratio (volume filled with air/total filter volume when not in operation), specific surface area (biofilter surface/biofilter volume) and the type of flow that the shape of the filter media allows across the biofilter (vertical flow, random flow or cross flow) (Kamstra et al., 1998)**.**

Temperature was the last factor selected for its great influence on the speed of chemical reactions (based on the Van't Hoff-Arrhenius equation) and bacterial growth and therefore the huge influence on biofilter performance. Some examples of papers analyzing the impact of temperature on the TAN removal in trickling filters include the experiments of Zhu and Chen (2002) and Lyssenko and Wheaton (2006). Zhu and Chen (2002) reported that the influence of temperature on nitrification speed was lower than predicted by the Van't Hoff-Arrhenius equation, but still had a considerable influence.

The aim of this study is to select the best set of these process parameters for achieving the best possible performance of nitrifying trickling filters, but also to provide information on the performance of the efficiency of biofilters under a wide range of conditions, depending on fish production plans. The three TAN loads simulated (3, 6 and 9 gTAN m^{-3} of tank water day^{-1}) are in accordance with TAN loads estimated for turbot aquaculture facilities depending on growth state (based on the study of Dosdat *et al.* (1996) and in which density is 7.5, 11 and 22.5 kg m^{-3}. The three temperature values (15ºC, 19ºC and 24ºC) set were in accordance with water temperatures established in recirculating aquaculture systems to produce several species, and to mean temperatures reached in the sea at certain time periods of the year for a sea-cage aquaculture facility. With regard to the filter media, trickling filters were traditionally constructed using rocks, but today most filters use plastic media, because of their low weight, high specific surface area and high void ratio (>90%). In the present experiment, three plastic materials were selected for their positive characteristics such as availability, easy manipulation (low weight) and price. Hitherto, to our knowledge no study has been made to determine the influence of these filter media on the efficiency of trickling filters.

In summary, this paper presents a tri-factorial study where the influence of three process parameters (temperature, filter media and TAN load) on the performance of trickling filters is assessed. Influence of each one of them are determined, but also the influence of the combination of process parameters on the achievement of certain N-TAN removal rates.

2. Material and methods

2.1. Tanks and biofilters

The system was composed by six 500 L tanks connected to six trickling filters. The water flows from the drainpipe of these tanks to the top of the filter by a peristaltic pump (Oceanrunner® OR3500, Aqua-Medic®, Bissendorf, Germany). The height from the bottom of the drainpipe to the top of the filter was close to 2.5 m. At the top of the filter the water was dispersed by a series of several holes in the pipe, to ensure the soaking of the entire surface area of the filter medium, contained in a home-made cube. The base of this home-made cube had several holes, from which water was returned to the tank. Biofilters were designed to establish an equal hydraulic surface loading rate of 12 m^3 m^{-2} $hour^{-1}$ in all of them. The water flow provided by the pump was 2400 L h^{-1}, minus friction and elevation losses.

The three different filter media used in this paper are Bactoballs® (Aqua-Medic®, Bissendorf, Germany), MECHpro® rings (Eheim®, Deizisau, Germany) and type A Biofill® (Bioscience, Inc., Allentown, USA) Two biofilters each contain the same type of filter medium, in different amounts, leading to six biofilters with duplicate filter media, although with a different biofiltration area. The characteristics of the biofilters are shown in Table 3.1, and were not modified until the end of the study. The reason for adding different amounts of filter medium to two different biofilters containing the same type of filter medium was to observe the differences in the performance. Nevertheless, when expressing the N-TAN removal rate the results are standardized by the biofiltration area.

Table 3.1. Characteristics of biofilters

	FILTER MEDIA TYPE	***SPECIFIC SURFACE (m^2m^{-3})***	***FILTER MEDIA VOLUME (m^3)***	***FILTER AREA (m^2)***
FILTER 1	*Bactoballs®*	*300*	*0.017*	*5.1*
FILTER 2	*MECHpro®*	*1150*	*0.009*	*10.35*
FILTER 3	*Type A Biofill®*	*180*	*0.025*	*4.5*
FILTER 4	*Bactoballs®*	*300*	*0.050*	*15.0*
FILTER 5	*MECHpro®*	*1150*	*0.050*	*57.5*
FILTER 6	*Type A Biofill®*	*180*	*0.050*	*9.0*

The water in the tanks was manufactured in the laboratory, adding the corresponding amount of salt (Sea salt for human consumption, Salinera Española S.A., San Pedro del Pinatar, Murcia, Spain), 18.5 kg, to 500 L of tap water, which was kept in one large water reservoir during at least one day to remove chlorine. In that way, it is ensured that no organic matter or microorganisms are added to the bulk water, assuring the continuous biofilter performance.

There was also no supplemental aeration: the constant falling of the water from the cube to the tank was enough to achieve an approximate constant oxygen concentration. No action was taken to adjust or compensate pH variations, above all, because those variations were very low all over the trials and neither was supplementary carbon added to the tanks.

2.2. Measurements

2.2.1. Trickling filter development

Filter development took up to four weeks until it was considered that the full-grown status had been reached. In this period, 1.5 g of NH_4Cl was added daily to each tank, and the TAN concentration was measured at 9:00 a.m. The objective was to reduce the TAN concentration to 0 mg L^{-1} after 24 hours had passed since its addition. When the measured TAN was 0 mg L^{-1} at 9:00 a.m. along several days, the trial began.

2.2.2. Trial measurements

TAN and oxygen concentrations, pH and temperature of the effluent water of the six biofilters were measured in duplicated tanks every two hours along a 24-hour period. 6 trials of 24 hours were performed in a period, simulating three TAN loads (3, 6 and 9 gTAN m^{-3} day^{-1}). Three periods were selected according to room temperature (one period in summer, one in autumn and one in winter). In summary, 18 trials were conducted. For every week, two trials were conducted, and an identical protocol was designed:

- Each Monday and Tuesday the bulk water of all tanks was manufactured to assure that the TAN was 0 at the beginning of the trials.
- On Wednesdays at 8:00 a.m. the first samples were taken, and after that the corresponding TAN dose was added, distributed for a few hours to simulate a long feeding period.
- On Thursdays at 8:00 a.m., after taking the corresponding samples from the first trial, the second trial began with the addition of a new equal dose to the tanks, and the second trial was conducted until the end of it on Fridays at 8:00 a.m.

A detailed protocol of the experiment can be seen in Table 3.2.

Table 3.2. Detailed experimental procedure

	Addition-depending protocol		
Hour	**3 gTAN m^{-3} day^{-1}**	**6 gTAN m^{-3} day^{-1}**	**9 gTAN m^{-3} day^{-1}**
8:00	Sampling followed by 0.9 g NH_4Cl addition	Sampling followed by 1.8 g NH_4Cl addition	Sampling followed by 2.7 g NH_4Cl addition
8:15	TAN measurement	TAN measurement	TAN measurement
10:00	Sampling followed by 2.25 g NH_4Cl addition	Sampling followed by 4.5 g NH_4Cl addition	Sampling followed by 6.75 g NH_4Cl addition
10:15	TAN measurement	TAN measurement	TAN measurement
12:00	Sampling followed by 1.35 g NH_4Cl addition	Sampling followed by 2.7 g NH_4Cl addition	Sampling followed by 4.05 g NH_4Cl addition
12:15	TAN measurement	TAN measurement	TAN measurement
14:00	Sampling every two hours from this moment until 8:00 of the following day and repeat	Sampling every two hours from this moment until 8:00 of the following day and repeat	Sampling every two hours from this moment until 8:00 of the following day and repeat

Mean temperature of the summer period (July-August) was established as 24.3ºC, mean temperature of the autumn period (September-October) was established as 19.0ºC, and mean temperature of the winter period (January-February) was established as 15.3ºC.

2.3. Equipment and chemical products

The TAN concentration and pH measurements were carried out by Orion® 4-Star Plus probe (ThermoScientific®, Waltham, Massachusetts, USA) together with ammonia and pH specific electrodes. Measurements of the TAN concentration were performed as described in García García *et al.* (2011). After the sample was taken, 100 µL of hydrochloric acid (J.T.Baker®, Avantor™, Central Valley, U.S.A.) were added and 1 mL of sodium hydroxide (Scharlau, Scharlab, Barcelona, España) was also added just before the ammonia ion selective electrode was used. The meter provided concentration of total ammonia nitrogen (NH_3 + NH_4^+).

Temperature and oxygen concentration measurements were carried out with a Handy Polaris® oximeter (OxyGuard®, Farum, Denmark). The probe package contained chemical products used for their preservation.

2.4. Data processing

TAN concentration results (mgTAN L^{-1}) presented were transformed into mass units (mgTAN), multiplying concentration by tank volume (500 L), and subsequently transformed into mg N-TAN (which is the quantity of nitrogen contained in the TAN molecule). That second transformation was made with the following equation:

$$N - TAN = TAN * \frac{molecular\ weight\ N}{molecular\ weight\ NH_4} \quad \text{(Eq. 1)}$$

The objective of measuring the concentration every two hours was to detect patterns in N-TAN concentration variation along a standard day of operation in an aquaculture farm, based in a specific feeding regime, as well as the maximum N-TAN concentration values and the time of maximum N-TAN removal. All results for each trial described a function that was estimated and quantified. A third-degree polynomial regression was thus performed for the series of results of the 24-hours trials. This model follows the typical pattern of a TAN or N-TAN daily variation in an aquaculture tank in which feeding is distributed for a relatively large period on the same time every day, and it has been described in literature (Eding et al., 2006), and can be used to estimate the approximate N-TAN value at a specific type provided that the process parameters are similar to the determinate model. To determinate if our results fitted to that model, replicates were averaged out, the regressions were made with these results and squared Rs were observed. Statgraphics also provided information concerning quality the of the model. To detect the period up to maximum N-TAN removal a derivative of the models was established. The minimum of that derivative indicated maximum N-TAN removal, which was added to the results.

To calculate N-TAN removal in one day (expressed in g of N-TAN removed divided by biofiltration area), the difference between the N-TAN (g) added to the tank and the N-TAN (g) value at t=24 h was calculated and divided by the biofiltration area, for each one of the six biofilters, 24-hour trials, temperature and TAN additions tested. When there were significant differences in the achievement of N-TAN removal rates depending on the value of an isolated process parameter (temperature and TAN load), simple regressions were performed with the objective of estimate these relations quantitatively. The type of simple regression (lineal for TAN load and exponential for temperature) was selected by observation of the data and literature review in case (Greiner and Timmons, 1998; Kamstra et al., 1998; Zhang et al., 2014; Zhu and Chen, 2002).

2.5. Statistics

All statistical analyses were made by Statgraphics® Centurion XVI for Windows®. One-way ANOVAS were made to evaluate the influence of each one of the process parameters on the biofilter performance. The combination of the influence of two process parameters on the biofilter performance were tested by multivariable ANOVAS. In both cases, the multiple range test was carried out by the Student-Newman-Keuls test. Third-degree polynomial regressions for the modeling of the N-TAN daily variation and lineal and exponential regressions (both simple and multiple) for the evaluation of the influence of process parameters on N-TAN removal rate were performed with Statgraphics®. Differences were considered significant at $P < 0.05$.

3. Results

3.1. pH and oxygen

pH and oxygen concentration remained quite constant throughout the entire experiment. No important changes along the 24 hour trials when the bacterial activity was at maximum point. Mean pH calculated was 8.26 ± 0.05 and mean oxygen concentration was 6.22 ± 0.18 mg L^{-1}.

3.2. N-TAN daily variation

In Figure 3.1, Figure 3.2 and Figure 3.3 is presented the N-TAN evolution along the day gathered by filter media, dose and period. The two series of data corresponding to the two biofilters that contain the same type of filter media in different biofiltration area are presented on the same chart, being *a1* the biofilters associated to tanks 1, 2, and 3 and *a2* the biofilters associated to tanks 4, 5 and 6. The equations of the individual third-degree polynomial regressions for each series of data are presented in Table 3.3. Summary of the third degree polynomial regressions with R2 and time of maximum N-TAN removal (g N-TAN removed m^{-2} day^{-1}), classified by biofilter, mean temperature and TAN load, as well as the time of maximum N-TAN removal.

Squared Rs ranged from 0.50 to 0.90 and every series of N-TAN concentration values fitted to a third degree polynomial regression according to Statgraphics®. In every measurement, the N-TAN concentration shows a peek between 6 and 8 hours and after

that it diminishes gradually until reaching a minimum around 20 hours. In several cases, the N-TAN concentration reached 0 at that point.

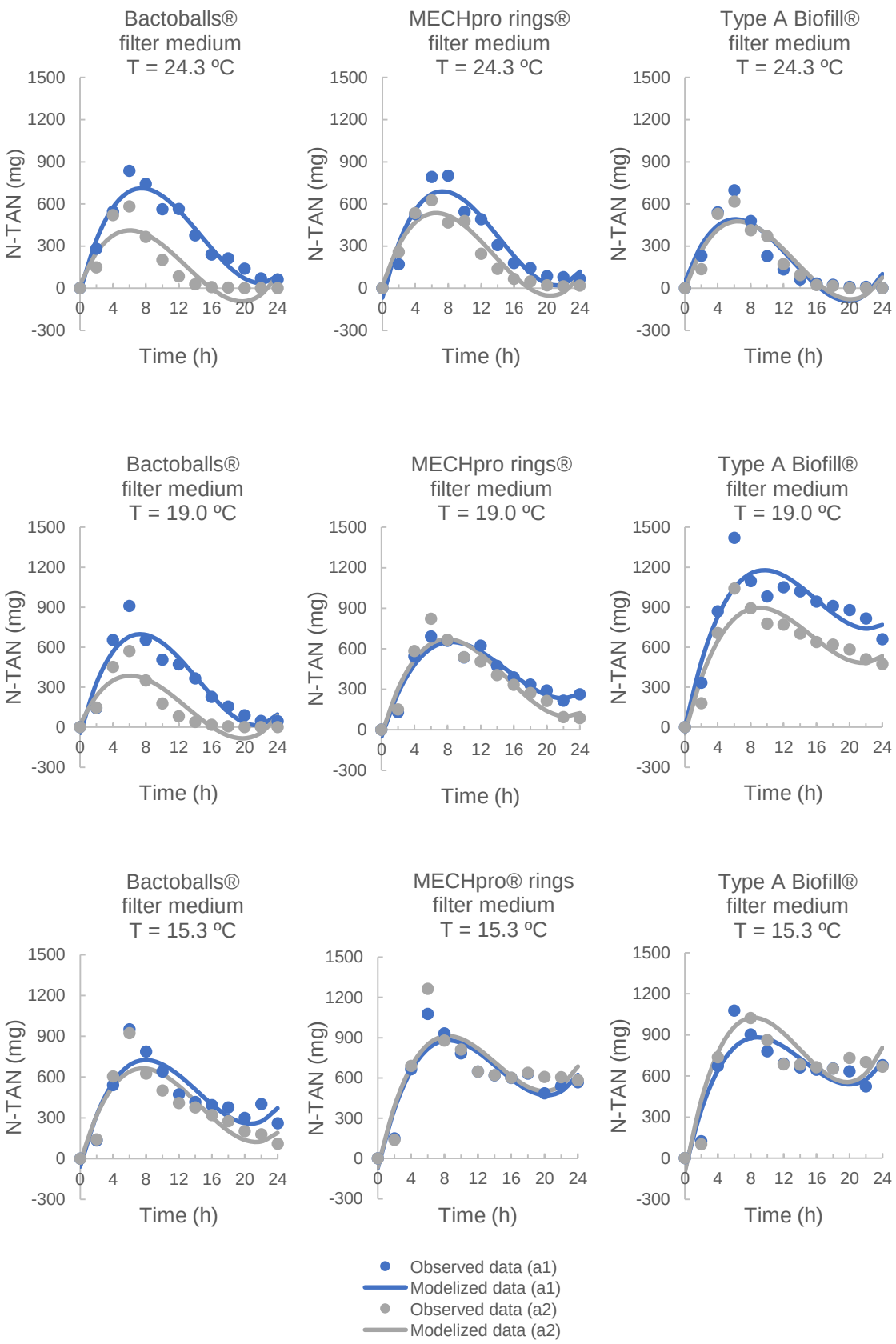

Figure 3.1. Summary of the daily N-TAN variation for every trial whose TAN load was 3 g m^{-3} day^{-1} (a1 and a2 stand for the two different biofiltration areas of the two biofilters containing the same filter media).

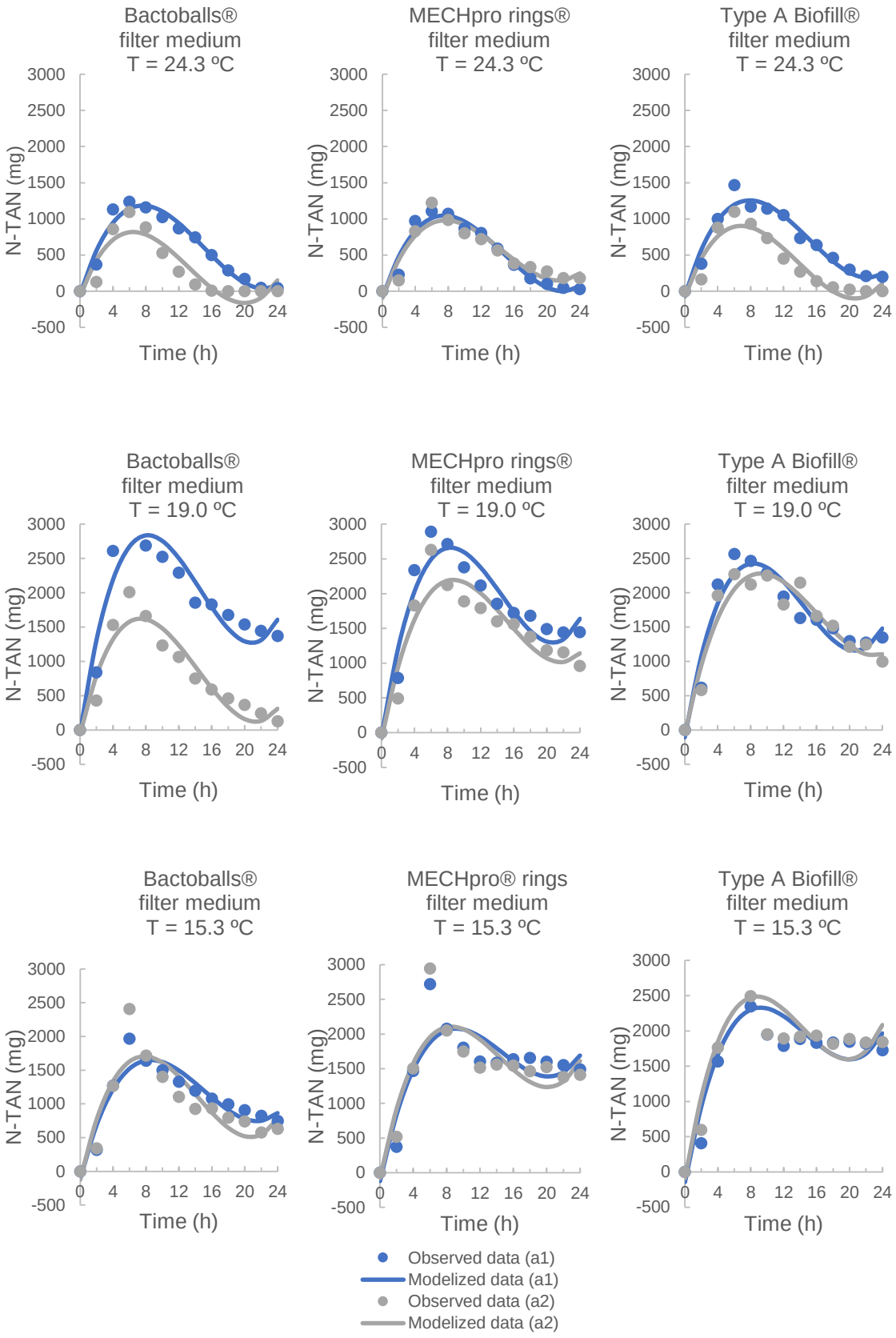

Figure 3.2. Summary of the daily N-TAN variation for every trial whose TAN load was 6 g m^{-3} day^{-1} (a1 and a2 stand for the two different biofiltration areas of the two biofilters containing the same filter media).

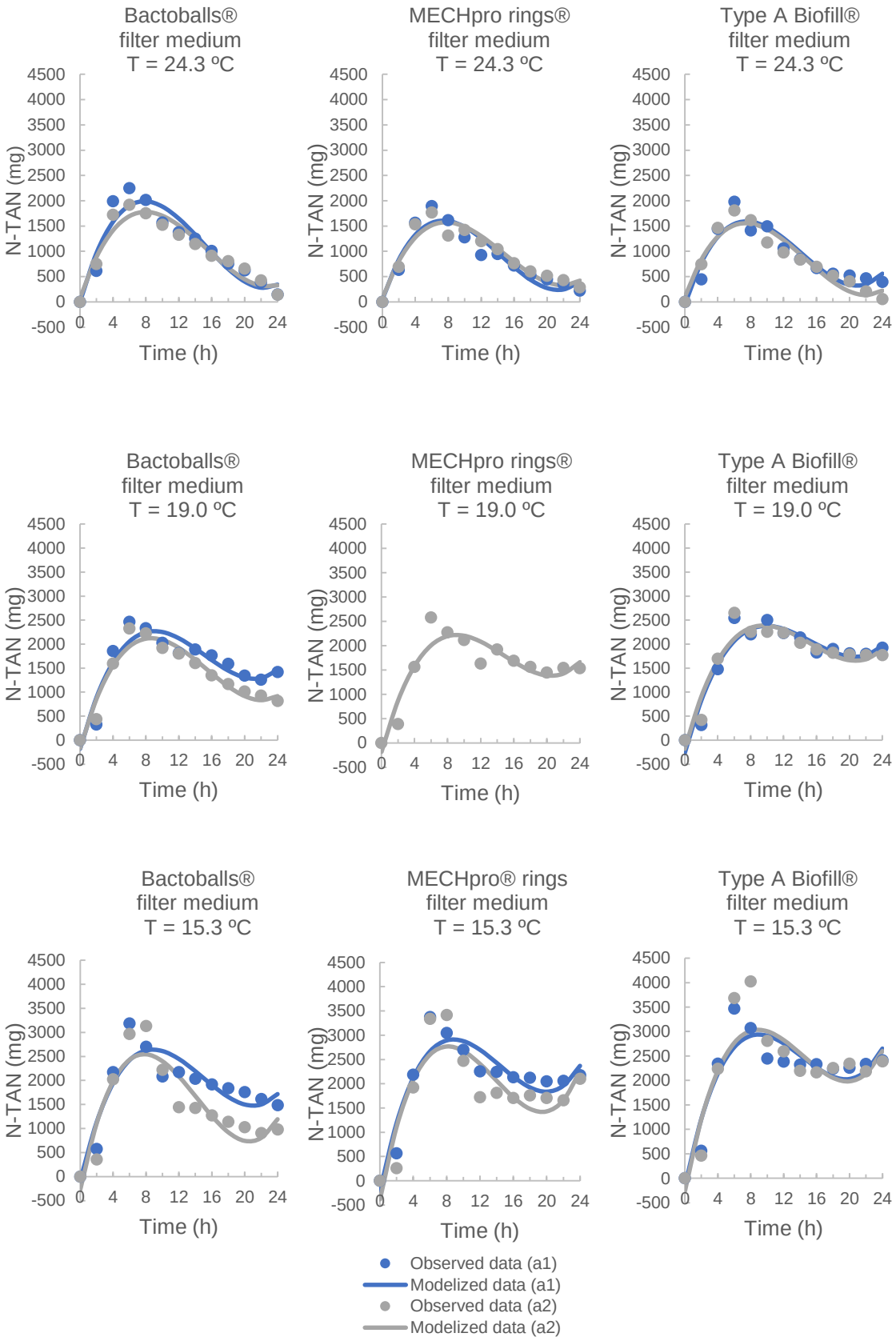

Figure 3.3. Summary of the daily N-TAN variation for every trial whose TAN load was 9 g m^{-3} day^{-1} (a1 and a2 stand for the two different biofiltration areas of the two biofilters containing the same filter media).

Table 3.3. Summary of the third degree polynomial regressions with R^2 and time of maximum N-TAN removal (g N-TAN removed m^{-2} day^{-1}), classified by biofilter, mean temperature and TAN load

TAN load = 3 g m^{-3} day^{-1}	**Summer period (Mean temperature = 24.3 ºC)**			
	Biofilter	N-TAN (mg) variation model	R^2 (%)	Max rN-TAN (g N-TAN removed m^{-2} day^{-1})
	1) Bactoballs® a1	N-TAN (t) = -18.61 + 220.63*t - 19.82*t^2 + 0.45*t^3	94.38	up to 14.6 h
	4) Bactoballs® a2	N-TAN (t) = 28.68 + 141.42*t - 15.32*t^2 + 0.40*t^3	77.31	up to 12.9 h
	2) MECHpro® a1	N-TAN (t) = -68.15 + 234.08*t - 21.55*t^2 + 0.50*t^3	91.55	up to14.3 h
	5) MECHpro® a2	N-TAN (t) = 19.20 + 176.50*t - 17.82*t^2 + 0.44*t^3	92.45	up to 13.8 h
	3) Type A Biofill® a1	N-TAN (t) = 41.87 + 163.70*t - 17.51*t^2 + 0.45*t^3	80.22	up to 13.0 h
	6) Type A Biofill® a2	N-TAN (t) = -5.80 + 167.43*t - 17.16*t^2 + 0.43*t^3	85.79	up to 13.3 h
	Autumn period (Mean temperature = 19.0 ºC)			
	Biofilter	N-TAN (mg) variation model	R^2 (%)	Max rN-TAN (g N-TAN removed m^{-2} day^{-1})
	1) Bactoballs® a1	N-TAN (t) = -44.56 + 229.50*t - 21.09*t^2 + 0.49*t^3	87.26	up to 14.3 h
	4) Bactoballs® a2	N-TAN (t) = 25.70 + 132.60*t - 14.28*t^2 + 0.37*t^3	77.26	up to 12.9 h
	2) MECHpro® a1	N-TAN (t) = -50.24 + 190.10*t - 15.58*t^2 + 0.34*t^3	91.18	up to 15.2 h
	5) MECHpro® a2	N-TAN (t) = -33.92 + 201.72*t - 17.25*t^2 + 0.38*t^3	88.59	up to 15.1 h
	3) Type A Biofill® a1	N-TAN (t) = -4.56 + 286.09*t - 21.23*t^2 + 0.44*t^3	83.48	up to 16.0 h
	6) Type A Biofill® a2	N-TAN (t) = -46.09 + 242.61*t - 19.05*t^2 + 0.41*t^3	86.34	up to 15.4 h
	Winter period (Mean temperature = 15.3 ºC)			
	Biofilter	N-TAN (mg) variation model	R^2 (%)	Max rN-TAN (g N-TAN removed m^{-2} day^{-1})
	1) Bactoballs® a1	N-TAN (t) = -59.07 + 226.05*t - 19.65*t^2 + 0.46*t^3	77.40	up to 14.2 h
	4) Bactoballs® a2	N-TAN (t) = -23.13 + 204.14*t - 18.15*t^2 + 0.42*t^3	78.15	up to 14.4 h
	2) MECHpro® a1	N-TAN (t) = -72.40 + 260.74*t - 21.78*t^2 + 0.50*t^3	81.70	up to 14.5 h
	5) MECHpro® a2	N-TAN (t) = -66.34 + 269.93*t - 22.73*t^2 + 0.53*t^3	71.93	up to 14.3 h
	3) Type A Biofill® a1	N-TAN (t) = -76.58 + 256.10*t - 20.99*t^2 + 0.49*t^3	81.38	up to 14.3 h
	6) Type A Biofill® a2	N-TAN (t) = -97.28 + 313.87*t - 26.80*t^2 + 0.64*t^3	65.40	up to 14.0 h
TAN load = 6 g m^{-3} day^{-1}	**Summer period (Mean temperature = 24.3 ºC)**			
	Biofilter	N-TAN (mg) variation model	R^2 (%)	Max rN-TAN (g N-TAN removed m^{-2} day^{-1})
	1) Bactoballs® a1	N-TAN (t) = -39.57 + 363.65*t - 32.05*t^2 + 0.71*t^3	96.11	up to 14.9 h
	4) Bactoballs® a2	N-TAN (t) = -61.01 + 305.82*t - 31.40*t^2 + 0.79*t^3	81.96	up to 13.2 h
	2) MECHpro® a1	N-TAN (t) = -87.60 + 341.57*t - 30.59*t^2 + 0.69*t^3	94.73	up to 14.7 h
	5) MECHpro® a2	N-TAN (t) = -95.15 + 317.26*t - 28.04*t^2 + 0.64*t^3	87.16	up to 14.5 h
	3) Type A Biofill® a1	N-TAN (t) = -65.79 + 379.95*t - 32.60*t^2 + 0.72*t^3	94.30	up to 15.1 h
	6) Type A Biofill® a2	N-TAN (t) = -81.06 + 323.34*t - 31.44*t^2 + 0.76*t^3	89.34	up to 13.8 h
	Autumn period (Mean temperature = 19.0 ºC)			
	Biofilter	N-TAN (mg) variation model	R^2 (%)	Max rN-TAN (g N-TAN removed m^{-2} day^{-1})
	1) Bactoballs® a1	N-TAN (t) = -23.83 + 800.62*t - 67.80*t^2 + 1.55*t^3	87.47	up to 14.5 h
	4) Bactoballs® a2	N-TAN (t) = -54.44 + 516.50*t - 47.30*t^2 + 1.10*t^3	87.90	up to 14.3 h
	2) MECHpro® a1	N-TAN (t) = -51.32 + 750.50*t - 63.07*t^2 + 1.45*t^3	90.22	up to 14.5 h

	5) MECHpro® a2	N-TAN (t) = -84.62 + 606.86*t - 48.97*t^2 + 1.08*t^3	85.18	up to 15.2 h
	3) Type A Biofill® a1	N-TAN (t) = -106.80 + 700.91*t - 58.87*t^2 + 1.35*t^3	91.08	up to 14.5 h
	6) Type A Biofill® a2	N-TAN (t) = -61.78 + 584.78*t - 44.26*t^2 + 0.91*t^3	91.52	up to 16.2 h
	Winter period (Mean temperature = 15.3 ºC)			
	Biofilter	N-TAN (mg) variation model	R^2 (%)	Max rN-TAN (g N-TAN removed m^{-2} day^{-1})
	1) Bactoballs® a1	N-TAN (t) = -114.29 + 472.19*t - 38.39*t^2 + 0.85*t^3	86.21	up to 15.1 h
	4) Bactoballs® a2	N-TAN (t) = -125.14 + 535.96*t - 47.28*t^2+ 1.10*t^3	76.27	up to 14.3 h
	2) MECHpro® a1	N-TAN (t) = -118.97 + 565.77*t - 44.95*t^2+ 1.02*t^3	76.08	up to 14.7 h
	5) MECHpro® a2	N-TAN (t) = -76.17 + 593.72*t - 49.39*t^2+ 1.15*t^3	71.77	up to 14.3 h
	3) Type A Biofill® a1	N-TAN (t) = -144.08 + 635.76*t - 50.47*t^2+ 1.15*t^3	73.92	up to 14.6 h
	6) Type A Biofill® a2	N-TAN (t) = -90.65 + 690.27*t - 56.81*t^2+ 1.33*t^3	73.92	up to 14.3 h
TAN load = 9 g m^{-3} day^{-1}	**Summer period (Mean temperature = 24.3 ºC)**			
	Biofilter	N-TAN (mg) variation model	R^2 (%)	Max rN-TAN (g N-TAN removed m^{-2} day^{-1})
	1) Bactoballs® a1	N-TAN (t) = 11.73 + 574.22*t - 49.63*t^2 + 1.10*t^3	89.24	up to 15.2 h
	4) Bactoballs® a2	N-TAN (t) = 83.86 + 482.61*t - 40.87*t^2 + 0.88*t^3	92.23	up to 14.1 h
	2) MECHpro® a1	N-TAN (t) = 46.23 + 477.2*t - 43.43*t^2 + 1.01*t^3	89.00	up to 14.4 h
	5) MECHpro® a2	N-TAN (t) = 70.17 + 441.23*t - 38.68*t^2 + 0.87*t^3	91.80	up to 14.8 h
	3) Type A Biofill® a1	N-TAN (t) = -55.47 + 498.85*t - 45.14*t^2 + 1.06*t^3	87.13	up to 14.2 h
	6) Type A Biofill® a2	N-TAN (t) = 93.02 + 445.57*t - 40.25*t^2 + 0.91*t^3	91.09	up to 14.7 h
	Autumn period (Mean temperature = 19.0 ºC)			
	Biofilter	N-TAN (mg) variation model	R^2 (%)	Max rN-TAN (g N-TAN removed m^{-2} day^{-1})
	1) Bactoballs® a1	N-TAN (t) = -181.58 + 632.37*t - 49.90*t^2+ 1.10*t^3	87.37	up to 15.2 h
	4) Bactoballs® a2	N-TAN (t) = -166.51 + 608.98*t - 48.96*t^2+ 1.06*t^3	92.20	up to 15.4 h
	5) MECHpro® a2	N-TAN (t) = -184.92 + 621.65*t - 49.44*t^2+ 1.11*t^3	85.41	up to 14.9 h
	3) Type A Biofill® a1	N-TAN (t) = -272.47 + 636.84*t - 47.52*t^2 + 1.04*t^3	90.77	up to 15.4 h
	6) Type A Biofill® a2	N-TAN (t) = = -181.12 + 633.43*t - 48.28*t^2+ 1.06*t^3	90.19	up to 15.2 h
	Winter period (Mean temperature = 15.3 ºC)			
	Biofilter	N-TAN (mg) variation model	R^2 (%)	Max rN-TAN (g N-TAN removed m^{-2} day^{-1})
	1) Bactoballs® a1	N-TAN (t) = -88.08 + 717.11*t - 57.58*t^2 + 1.28*t^3	81.27	up to 15.0 h
	4) Bactoballs® a2	N-TAN (t) = -280.72 + 832.01*t - 73.70*t^2 + 1.73*t^3	80.79	up to 15.2 h
	2) MECHpro® a1	N-TAN (t) = -204.00 + 827.84*t - 67.62*t^2 + 1.57*t^3	85.96	up to 14.4 h
	5) MECHpro® a2	N-TAN (t) = -384.02 + 897.28*t - 77.96*t^2 + 1.88*t^3	76.57	up to 13.8 h
	3) Type A Biofill® a1	N-TAN (t) = -154.92 + 813.87*t - 66.21*t^2 + 1.55*t^3	83.19	up to 14.3 h
	6) Type A Biofill® a2	N-TAN (t) = -258.59 + 871.40*t - 71.08*t^2 + 1.66*t^3	81.92	up to 14.3 h

Relation between TAN load and mainly N-TAN mass measured in the tanks throughout the trials is evident. The higher the TAN load was, the higher are the peaks of N-TAN mass. In addition, in Figure 3.1, Figure 3.2 and Figure 3.3 is shown a relation between temperature and N-TAN mass. It is observed that in the higher TAN loads (6 and 9 g m^{-3} day^{-1}) for type A Biofill® over all, that, when the addition is the same, the combination of trials performed in the summer period (mean temperature = 24.3ºC) led to lower peaks

of N-TAN than in the autumn period (mean temperature = 19.0ºC) and in the winter period (mean temperature = 15.3ºC).

3.3. Influence of individual process parameters

3.3.1. Influence of the filter media type on the N-TAN removal rate

Table 3.4. Mean and standard error of the N-TAN removal rates in relation to the filter media type. Different superscripts in the same column indicate statistical differences (p-value<0.05).

Filter media type	n	Mean rN-TAN (g N-TAN removed m^{-2} day^{-1})	Standard error
MECHpro®	34	0.078^{b}	0.024
Type A Biofill®	36	0.223^{a}	0.024
Bactoballs®	36	0.235^{a}	0.024

Table 3.4 shows the mean of N-TAN removal rates achieved in relation to the filter media type, for every trial in every period. Bactoballs® was the filter medium type that let the biofilters which contained them achieve the highest mean efficiency in N-TAN removal (rN-TAN = 0.24 g N-TAN removed m^{-2} day^{-1}), although there were no significant differences with the mean N-TAN removal rate achieved by biofilters containing type A Biofill® (rN-TAN = 0.22 g N-TAN removed m^{-2} day^{-1}).

3.3.2. Influence of the temperature on the N-TAN removal rate

Table 3.5. Mean and standard error of the N-TAN removal rates in relation to the mean temperature. Different superscripts in the same column indicate statistical differences (p-value<0.05).

Temperature (ºC)	n	Mean rN-TAN (g N-TAN removed m^{-2} day^{-1})	Standard error
15.3	36	0.126^{b}	0.024
19.0	34	0.151^{b}	0.025
24.3	36	0.263^{a}	0.024

Table 3.5 shows the mean of N-TAN removal rates achieved in relation to the temperature of the water. The highest mean N-TAN removal rate, 0.26 g N-TAN removed m^{-2} day^{-1}, was achieved in the summer period (24.3ºC). However, there were no significant differences between the mean of N-TAN removal rates achieved in the autumn period and in the winter period.

3.3.3. Influence of the TAN load on the N-TAN removal rate

Table 3.6. Mean and standard error of the N-TAN removal rates in relation to TAN added to tank water. Different superscripts in the same column indicate statistical differences (p-value<0.05).

TAN load (g m^{-3} day^{-1})	n	Mean rN-TAN (g N-TAN removed m^{-2} day^{-1})	Standard error
3	36	0.105^{c}	0.023
6	34	0.174^{b}	0.023
9	36	0.269^{a}	0.024

Table 3.6 shows the mean of N-TAN removal rates achieved in relation to the TAN load. Every increase on addition produced a significantly higher N-TAN removal rate. The highest mean N-TAN removal rate was 0.27 g N-TAN removed m^{-2} day^{-1}, at an TAN load of 9 g m^{-3} day^{-1} per day.

3.4. Influence of the combination of process parameters

In Figure 3.4 mean N-TAN removal rates achieved for each possible combination of process parameters are presented, with the standard error of the multivariable ANOVA. In Table 3.7 is displayed a summary of the significance of the relation of values of a certain process parameter and N-TAN removal rates, when the remaining two process parameters were fixed. In the case of significant differences, a simple regression (linear or exponential) is performed and the regression equation and squared-R is presented.

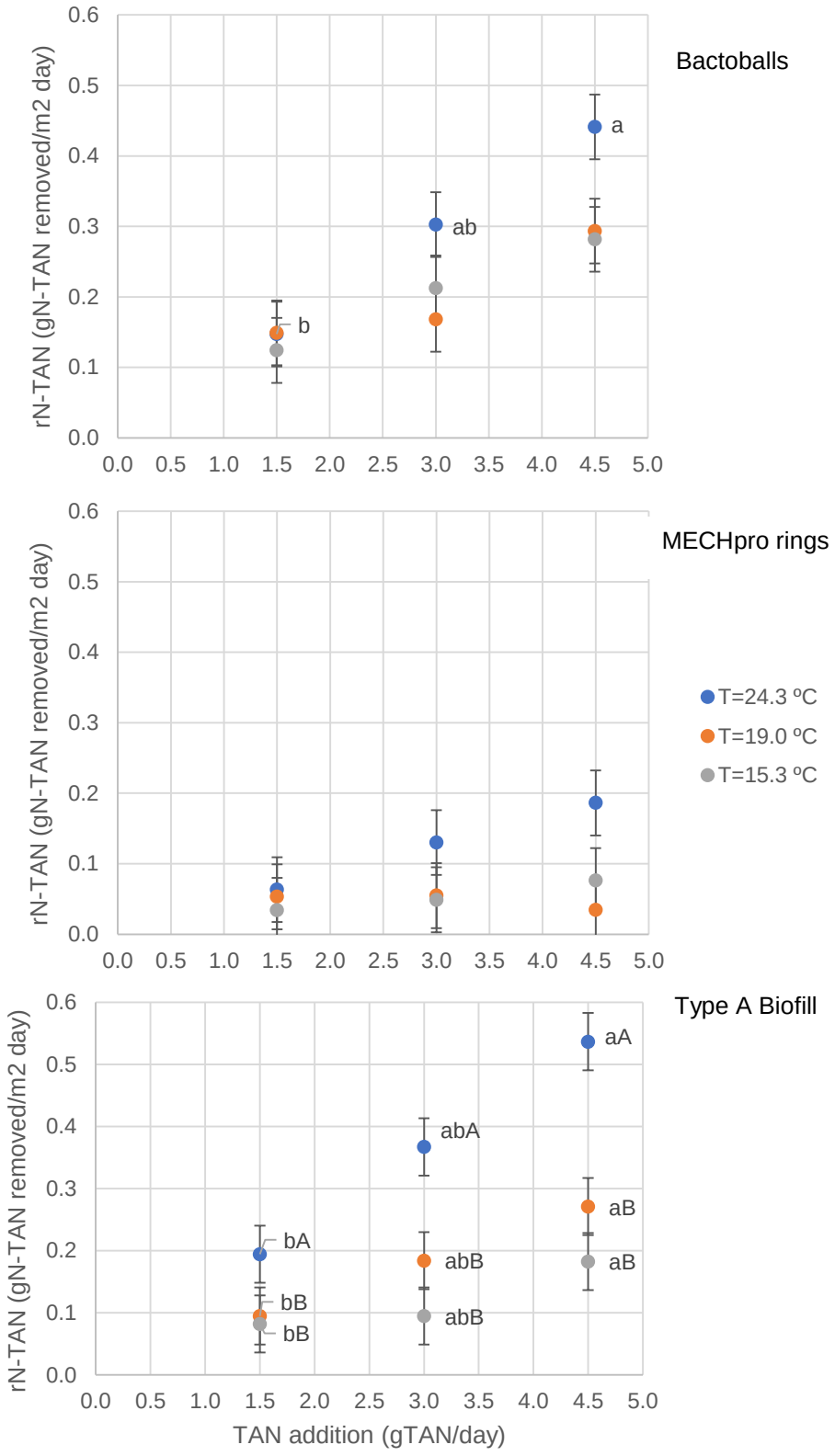

Figure 3.4. Mean N-TAN removal rates (±SEM: pooled standard error of the mean) achieved by biofilters classified by filter medium under fixed mean temperature and TAN production conditions. Small letters indicate statistical differences (p-value<0.05) on the N-TAN removal rates achieved depending on the N-TAN influent concentration whilst capital letters indicate significant differences on N-TAN removal rates achieved depending on the mean temperature.

Table 3.7. Significance of the differences of N-TAN removal rates achieved depending on the value of a certain process parameter. Differences were considered significant at p-value<0.05.

Filter medium	Fixed temperature (ºC)	Significant differences depending on TAN load	Regression / Squared-R
Bactoballs®	24.3	Yes	$y=0.098x+0.003$ $R^2=0.9989$
	19.0	No	
	15.3	No	
	Fixed TAN load (g TAN/m^3 day)	Significant differences depending on temperature	Regression / Squared-R
	3	No	
	6	No	
	9	No	
	Fixed temperature (ºC)	Significant differences depending on TAN load	Regression / Squared-R
MECHpro® rings	24.3	No	
	19.0	No	
	15.3	No	
	Fixed TAN load (g TAN/m^3 day)	Significant differences depending on temperature	Regression / Squared-R
	3	No	
	6	No	
	9	No	
	Fixed temperature (ºC)	Significant differences depending on TAN load	Regression / Squared-R
Type A Biofill®	24.3	Yes	$y=0.1141x+0.0239$ $R^2=1$
	19.0	Yes	$y=0.0587x+0.0073$ $R^2=1$
	15.3	Yes	$y=0.0335x+0.0192$ $R^2=0.8433$
	Fixed TAN load (g TAN/m^3 day)	Significant differences depending on temperature	Regression / Squared-R
	3	Yes	$y=0.0167*e^{0.0987x}$ $R^2=0.933$
	6	Yes	$y=0.0101*e^{0.1491x}$ $R^2=0.9918$
	9	Yes	$y=0.0284*e^{0.1204x}$ $R^2=0.9974$

These results combined with the simple ANOVAS results, suggest a poor performance of biofilters containing MECHpro® rings filter medium. No combination of temperature and TAN load led to a mean N-TAN removal rate comparable to mean N-TAN removal rates achieved by Bactoballs® and Type A Biofill® biofilters. In MECHpro® rings biofilters there were not any significant differences in mean N-TAN removal rates when an isolated process parameter (TAN load or temperature) was modified.

For Bactoballs®, although the global mean N-TAN removal rate (0.24 g N-TAN removed m^{-2} day^{-1}) is slightly higher than the global mean N-TAN removal rate for Type A Biofill® (0.22 g N-TAN removed m^{-2} day^{-1}), the maximum N-TAN removal rate (0.54 g N-TAN removed m^{-2} day^{-1}) was achieved by the biofilters that contained Type A Biofill® when operating at a mean temperature of 24.3ºC and a TAN load of 9 g m^{-3} day^{-1}. In the same conditions, mean N-TAN removal rate achieved by biofilters that contained Bactoballs® was 0.44 g N-TAN removed m^{-2} day^{-1}, which was nevertheless the maximum N-TAN removal rate achieved by these biofilters. Temperature did not show a correlation with rN-TAN for Bactoballs® biofilters in any TAN load. In the case of Bactoballs®, TAN load was only correlated with rN-TAN in the highest temperature. However, when Type A Biofill® was used, temperature (in exponential fitting) and also TAN load (with linear fitting) showed high correlation with N-TAN removal rates.

4. Discussion

TAN concentration along the trial followed an identical pattern for every tank/biofilter tested. This pattern was easily identified with the ammonia concentration variation during a day in an aquaculture farm as simulated by the trails. This N-TAN variation is easily portrayed as a third degree polynomial model: N-TAN rapidly increases as the excretion of NH_4^+ by the fish takes place (reaching a peak at 6-8 hours) and starts diminishing right after the end of the feeding period because no more NH_4^+ is produced and the biofilter slowly start to eliminate ammonia in an increasing efficiency up to a maximum removal around 13-14 hours. Finally, ammonia reaches its lowest level (ideally 0) until it increases rapidly again with the next feeding period. Similar polynomial models (second degree or fourth degree) didn't fit adequately with N-TAN concentration values obtained, either because squared Rs were too low or because p-values of the third degree terms were higher than 0.05, and thus statistically not significant.

The values of N-TAN concentration are however related to the different TAN loads (3, 6 and 9 g TAN per m^3 of tank water and day). Other differences between biofilters performances (based on filter media type or mean temperature) were noticed in the amount of TAN concentration. However, the stages of high TAN concentration lasted for a similar time. The mean N-TAN removal rate of all the performed trials was 0.18 g N-TAN removed m^{-2} day^{-1}, equivalent to 0.23 g TAN removed m^{-2} day^{-1}, although mean N-TAN removal rates ranged from 0.04 g TAN removed m^{-2} day^{-1} to 0.69 g TAN removed

m^{-2} day^{-1}. These values are consistent with the range of TAN removal rates reported by other authors and reviewed by Crab *et al.* (2007), which range from 0.16 and 1.1 g TAN removed m^{-2} day^{-1}.

In several trials biofilter performance surpassed initial estimation and all TAN added to the corresponding tank was totally consumed earlier than the end of the trials, in biofilters whose biofiltration area was elevated over all. In other cases, the monitoring of two biofilters with identical filter medium presented no differences in N-TAN concentration values although the amount of filter medium (and therefore a higher biofiltration area) was higher in one of them. Both cases indicate that a fraction of filter medium was not necessary to fill the nitrification requirements and the efficiency of those biofilters was not optimal. This is reflected on mean rN-TAN removal rates being slightly lower than expected. The best example is that the biofilter which presented the highest N-TAN removal rate (0.69 g TAN removed m^{-2} day^{-1}) was biofilter 3, which contained 4.5 m^2 of Type A Biofill® filter medium and was operated under a mean temperature of 24.3ºC and a TAN load of 4.5 g per day; whilst biofilter 6, which contained 9 m^2 of filter media, presented under the same operating conditions an N-TAN removal rate of 0.38 g TAN removed m^{-2} day^{-1}.

Results presented in Table 3.4, Table 3.5, Table 3.6 and Figure 3.4 prove that the values of the three operational parameters selected influence on the achievement of a wide range of N-TAN rates, although an isolated process parameter may not have an effect under certain circumstances. The effect of TAN load and temperature on the achievement of N-TAN removal rates, when observed, can be estimated quantitatively. Results pointed out that, when present, correlation of TAN load and N-TAN removal rate was lineal, while correlation of N-TAN removal rate and temperature was exponential. This is also similar to what is found in literature (Zhang et al., 2014). In this study every increase of TAN load lead to higher N-TAN removal rates, which was expected with high biofiltration areas as the ones that had the biofilters in this paper. Effect of temperature and TAN load is combined, and therefore the highest mean temperature and the highest TAN loads were the conditions where maximum N-TAN removal rate was achieved for almost every biofilter.

Figure 3.5 shows a modelling of N-TAN removal rates achieved according to the combination of the three process parameters. The modelling was prepared based on the

quantification of the effect of temperature and N-TAN influent concentration (by a multiple regression) for every filter media.

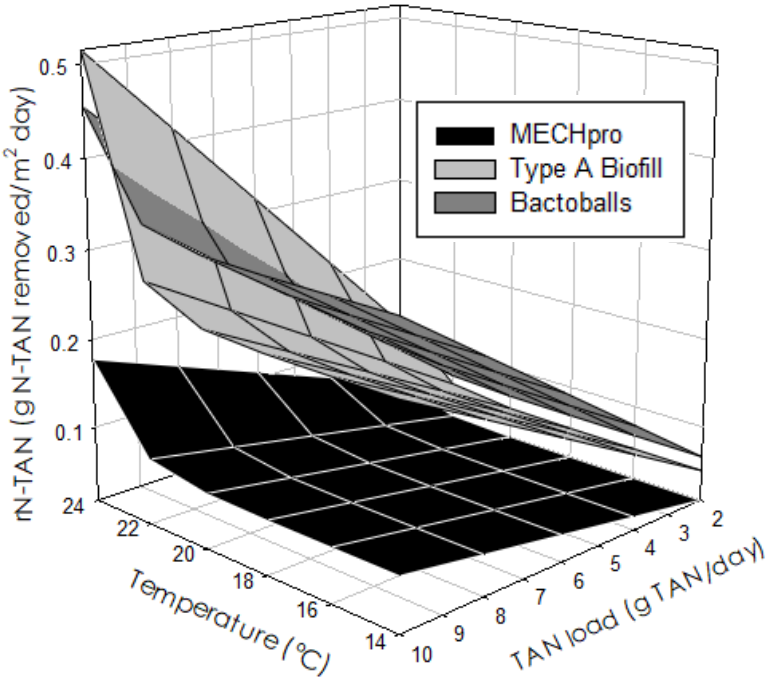

Figure 3.5. Three dimensional model showing quantitatively relations between N-TAN removal rates and the process parameters ammonia production and mean temperature for each one of the three filter media tested.

Results point out that MECHpro® rings, among the filter media tested in this study, led to the lowest N-TAN removal rates, and therefore it is not suggested in the construction of biofilters. The convenience of the usage of the other two filter media types largely depends on the operating conditions of the aquaculture plant. If a high mean water temperature and N-TAN concentration is allowed, Type A Biofill® presents the highest efficiency, but if the aquaculture plant requires low temperature Bactoballs® presents better performance.

The poor performance of MECHpro® rings could be explained by the small void ratio and by the water distribution through the biofilter rather than by the specific surface area, which is the highest among the filter media tested. Besides, very little differences are observed (in Figure 3.1, Figure 3.2 Figure 3.3) between the N-TAN (mg) measured in the biofilters effluent from the two biofilters containing the same filter media, despite the fact that one biofilter had approximately 5 times more filter media than the other, indicating that the nitrification reaction is not taking place in the entire surface area. The

other filter media types showed no significant difference in the biofilter performance in general, but under the best conditions one filter medium type (Type A Biofill®) led to a higher mean N-TAN removal rate.

It is shown that biofilters in which the TAN load and temperature was high present higher N-TAN removal rates. There is therefore needed to set the water or at least the biofilter water influent to a certain condition that are not always desirable. A high TAN load may suppose a higher risk of illness and death to fish, as N-TAN will certainly be present in culture water at high concentrations. Besides, it is not always possible to maintain a high fish density or a constantly elevated feeding rate. Temperature is also another problem for the turbot culture, considering that its optimal temperature range is lower than the 24ºC presented as the best temperature for maximizing biofilter performance. Results of N-TAN removal rates at lower TAN load rates and lower mean temperatures are presented for those cases, in which the modification of biofilters intrinsic properties (size, hydraulic loading, seriation…) may be the only solution.

CONCLUSIONS

N-TAN values followed an identical pattern for all biofilters set-ups, but at a certain TAN load differences in N-TAN maximum values are observed depending on temperature and filter media type. N-TAN removal rate (as a biofilter efficiency measure) is dependent on all three (filter medium type, TAN load and temperature) process parameters. The effect of a single process parameter when the others remain constant can be sometimes estimated quantitatively, linearly in case of the TAN load and exponentially in case of the mean temperature. The combination of the optimal values of these process parameters can lead to higher N-TAN removal rates in certain occasions, while in other cases the filter media type is the most determining factor and optimal values of the other process parameters does not imply a higher efficiency. In the same way less optimal values of temperature or TAN load did not suppose a significant reduction in biofilters efficiency when Bactoballs® were their filter media. On the other hand, conditions for maximizing biofilter performance can be compromised with fish welfare as higher TAN loads are required.

CHAPTER 4

Influence of temperature, ammonia load and hydraulic loading on the performance of nitrifying trickling filters for recirculating aquaculture systems

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ABSTRACT

In recirculating aquaculture systems, performance of nitrifying biofilters for total ammonia nitrogen (TAN) removal from the culture water and thus minimizing eutrophication depends on numerous elements of design. In this article the combined effect of three of these process parameters (temperature, hydraulic loading and TAN load) is evaluated. Ammonia removal rates (removed N-TAN divided by biofiltration area and day) were measured for every combination of five different temperatures, three different hydraulic loadings and three different ammonia loads. Every one of the process parameters were influential on nitrification rates and the lowest process parameters values corresponded with significantly lower N-TAN removal rates. A significantly higher mean N-TAN removal rate (0.241 gN-TAN removed m^{-2} day^{-1}) was found for the combination of the highest water temperature (27 ºC), the highest hydraulic loading (11 m^3 m^{-2} h^{-1}) and the highest TAN load (9 gTAN m^{-3} day^{-1}), suggesting a positive synergy of the three process parameters on the achievement of greater biofilter performances.

1. Introduction

Intensive aquaculture requires a meticulous waste treatment planning, especially in indoor recirculating aquaculture systems (RAS) (Piedrahita, 2003; Van Rijn, 2013). Wastes of the aquaculture industry include fecal matter, uneaten food, and dissolved carbonous, nitrogenous and phosphorous compounds that accumulate rapidly in the production units, in particular in RAS with high hydraulic retention time (Davidson et al., 2009), and filtration and purification systems must be implement to prevent fish health and welfare issues. Additionally, it is imperative this treatment before water is discharged to prevent environmental impacts (Van Rijn, 2013).

Ammonia is one of the key water quality parameters to be controlled in recirculating aquaculture. It is generated and excreted as an end product of the deamination of free amino acids taken in the fish diet with the purpose of generating energy (Dosdat et al., 1996). Fish diets in aquaculture usually contain a high protein content. As a result, excretion of ammonia is quite high and is one of the chemical compounds that most quickly accumulates in water after the feeding period (Engin et al., 2013; Godoy-Olmos et al., 2016; Obirikorang et al., 2015; Riche, 2015). Ammonia is toxic to fish, most notably in unionized form, causing nervous system-related malfunctions (Randall and Tsui, 2002; Tomasso, 1994). Several researchers have conducted toxicity experiments that

demonstrate negative effects of low concentrations of NH_3 in both survival and growth rate for several species (Foss et al., 2009, 2004; Lemarié et al., 2004). Because of that the guidelines for good aquaculture practices include recommendations on levels of unionized ammonia as low as 0.0125 mg L^{-1} (Chen et al., 2006).

Nitrifying biofilters are installed in recirculating aquaculture systems to reduce this ammonia concentration and transform it into the less toxic nitrate in two phases: (1) conversion of ammonia to nitrite and (2) conversion of nitrite to nitrate. Both conversions have been thoroughly studied (Losordo and Westers, 1994; Sharma and Ahlert, 1977). The theoretical rate at which ammonia is eliminated follows a Monod-type equation directly dependent on the ammonia concentration if enough oxygen and $CaCO_3$ (limiting factors) are provided up until a certain limit (Bovendeur et al., 1987). However, the exact nitrification rate depends on multiple process parameters (besides the dissolved oxygen and $CaCO_3$ concentration described above), which and include filter media, TAN concentration in the biofilter influent, temperature, hydraulic loading, organic matter and pH (Chen et al., 2006; Eding et al., 2006).

Some of these process parameters require a compromise between maximizing biofilter performance and improving fish yield. For instance, an adequate temperature is crucial for maximizing the latter (Abbink et al., 2012; Person-Le Ruyet et al., 2004), but certain species that require cold water for survival and optimal growth such as turbot *(Scophthalmus maximus),* which leads to problems in biofilter performance because nitrification is inhibited at low temperatures (Zhu and Chen, 2002). The TAN load (defined by the amount of dissolved ammonia-nitrogenous compounds excreted by fish and carried out to the biofilter for removal) is a result of fish density (Wagner et al., 1995), fish size (Conklin et al., 2007) or protein intake (Ballestrazzi et al., 1994), different for every farming system. Hydraulic loading, which is defined as water flow divided by cross-sectional biofilter area (Eding et al., 2006), is an element of design of biofilters that can be increased via increasing water flow or shaping the biofilter increasing its height and reducing its diameter. Nijhof (1995) first suggested a positive effect of the hydraulic loading on ammonia removal rate explained by an improved wetting of the filter medium and a higher TAN load rate. On the other hand, increasing water flow or the height of the biofilter would result in an increase of the energetic costs (Kamstra et al., 1998). Therefore, the establishment of the optimal hydraulic loading is very important.

Due to the particular importance of these three process parameters being temperature, TAN loading and hydraulic loading, the combination of them have been tested in this paper, selecting five temperatures (16 ºC, 18 ºC, 21 ºC, 24 ºC and 27 ºC) three TAN loading rates (3 gTAN m^{-3} day^{-1}, 6 gTAN m^{-3} day^{-1} and 9 gTAN m^{-3} day^{-1}) and three hydraulic loadings (11 m^3 m^{-2} h^{-1}, 8 m^3 m^{-2} h^{-1} and 4 m^3 m^{-2} h^{-1}) and designing a three-factorial assay in which a range of N-TAN removal rates will be presented depending on potential fish production plans of aquaculture facilities.

2. Materials and methods

2.1. Experimental design

The experimental system was composed by independent six 330 L tanks connected to six independent trickling filters. Water was recirculated by peristaltic pumps (Oceanrunner® OR3500, Aqua-Medic®, Bissendorf, Germany). The height from the bottom of the drainpipe to the top of the filter was close to 2.5 m. Water was dispersed through the top area of the biofilters by means of “rain effect” assuring the whole surface was wet. Water flow provided by the pump was equal for the six subsystems (1.08 m^3 h^{-1}). A diagram of the experimental system is presented in Figure 4.1.

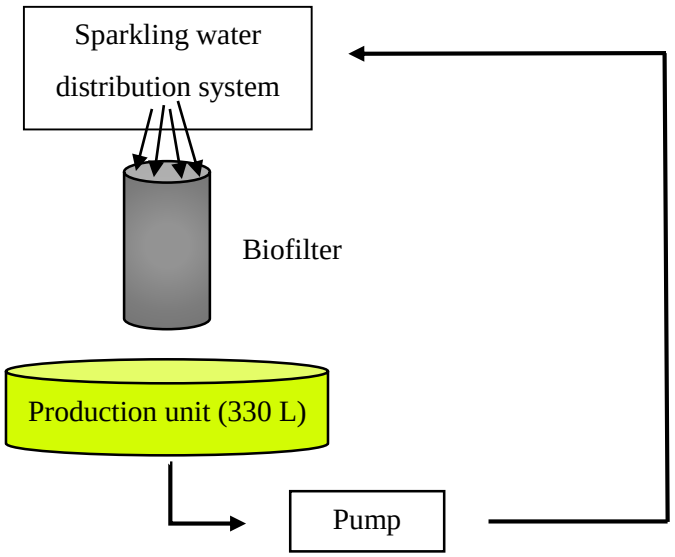

Figure 4.1. Diagram of the experimental system

The pilot-scale trickling filters, composed by plastic nets with cylindrical form and covered by plastic, were constructed in a way that three different hydraulic loadings could be

evaluated without altering water flow. Two identical biofilters per hydraulic loading were constructed. All six biofilters were filled with an equal volume (0.02 m^3) of Bactoballs® (plastic filter media, specific surface area 300 m^2 m^{-3}, Aqua-Medic®, Bissendorf, Germany). Detailed characteristics of biofilters are summarized in Table 4.1. Experiments were carried out in the Aquaculture Laboratory of the Universitat Politècnica de València.

Table 4.1. Characteristics of the six biofilters constructed for this research

	Biofilter 1	*Biofilter 2*	*Biofilter 3*	*Biofilter 4*	*Biofilter 5*	*Biofilter 6*
Water Flow (m^3 h^{-1})	1.08	1.08	1.08	1.08	1.08	1.08
Diameter (cm)	36	42	58	36	42	58
Height (cm)	22	16	8.4	22	16	8.4
Cross-sectional area (m^2)	0.10	0.14	0.26	0.10	0.14	0.26
Volume (m^3)	0.02	0.02	0.02	0.02	0.02	0.02
Hydraulic loading (m^3 m^{-2} h^{-1})	10.61	7.80	4.09	10.61	7.80	4.09

Water flowing in the tanks was artificial sea water manufactured in the laboratory composed of tap water with the addition of 12 Kg of sea salt (Sea salt for human consumption, Salinera Española S.A., San Pedro del Pinatar, Murcia, Spain) for each 330 L of water thus establishing a constant 37‰ salinity. Tap water was kept in a large reservoir for 24 hours to ensure the removal of chlorine. Water temperature was adjusted by internal heaters (EHEIM thermocontrol 300, Eheim GmbH, Deizisau, Germany), depending on the trial. Water was completely removed from every tank at the end of each trial, and replaced with ammonia-free water in which the next trials were carried out. This was done to ensure the least possible amount of interferences, as well as keeping pH and alkalinity identical for each biofilter performance measurement.

During the development of the microbial communities, 1.5 g of ammonium chloride was added daily to each tank. Alkalinity was measured every two days and sodium bicarbonate was added when needed to compensate the CO_3^- consumed by the bacteria. This procedure was followed up to six weeks, when the biofilter acquired full-grown status. TAN concentration was measured at 9:00 A.M. every day until it was 0 mg L^{-1} for several consecutive days. After that, performance measurements were initiated.

2.2. Performance measurements

An equal amount of ammonium chloride was added to each one of the six tanks following an identical protocol summarized in Table 4.2 which simulated a wide feeding period. Prior to the ammonia addition and every 2 hours until 24 hours since the addition samples were collected. The ammonia removal rates were calculated by difference between the ammonia concentration at the beginning (t=0) plus the ammonia that was added to the tanks minus the ammonia concentration at the end of periods of 24 hours, divided by biofiltration area. This protocol was performed twice in every tank (12 total) for each one of the three TAN load rates tested at a certain temperature. After that the temperature was increased to the next value and the protocol was carried out in the same manner.

TAN, nitrite, nitrate and oxygen concentrations, pH and temperature of the biofilter effluent of the six biofilters were measured every two hours along a 24-hour period. Alkalinity was also measured every six hours, beginning at 8:00 A.M. Protocol followed in every 24-hour sampling was identical, except for the change in the dosage of ammonium chloride.

Table 4.2. Protocol of the TAN addition and sampling during the trials.

Time (Hour)	*Addition-depending protocol*		
	3 gTAN m^{-3} day^{-1}	*6 gTAN m^{-3} day^{-1}*	*9 gTAN m^{-3} day^{-1}*
8:00	Sampling followed by 0.6g NH_4Cl addition	Sampling followed by 1.3g NH_4Cl addition	Sampling followed by 1.8g NH_4Cl addition
10:00	Sampling followed by 1.5g NH_4Cl addition	Sampling followed by 2.9g NH_4Cl addition	Sampling followed by 4.5g NH_4Cl addition
12:00	Sampling followed by 0.9g NH_4Cl addition	Sampling followed by 1.7g NH_4Cl addition	Sampling followed by 2.6g NH_4Cl addition
14:00	Sampling every two hours from this moment until 8:00 of the following day and repeat	Sampling every two hours from this moment until 8:00 of the following day and repeat	Sampling every two hours from this moment until 8:00 of the following day and repeat

2.3. Analytical protocols

Temperature and oxygen were measured by a Handy Polaris® oximeter (OxyGuard®, Farum, Denmark). pH was measured by Orion® 4-Star Plus probe (ThermoScientific®, Waltham, Massachusetts, USA) and its associated pH electrode. No further data processing was necessary, and values obtained are presented directly in the paper. TAN

and nitrite concentration were measured by spectrophotometry. The apparatus used in both determinations was a T60V UV-VIS spectrophotometer (PG Instruments, Leicester, UK). TAN concentration was determined with the indophenol method. Nitrite was measured by the Griess determination involving sulfanilamide and N-(1-naphthyl)ethylendiamine dihydrochloride. For the measuring of nitrate, a novel determination was carried out based on the use of vanadium(III) chloride as a reducing agent, first described in the paper of Miranda *et al.* (2001) and developed by Schnetger and Lehners (2014) for microplate readers. TAN and nitrite measurements were directly carried out as the samples were collected, and nitrate was measured for samples that were filtrated and then frozen at -18ºC up until the measurement day, which was performed with a Victor 1420 microplate reader (Perkin Elmer, formerly Wallac Oy, Massachussets, USA).

2.4. Statistics

Ammonia removal rates were calculated according to the following equation:

$$Ammonia\ removal\ rate = \frac{Tank\ volume\ (m^3)\ (N\text{-}TAN_{t=0} + N\text{-}TAN_{added} - N\text{-}TAN_{t=24})}{Biofiltration\ area\ (m^2)} \quad \text{(Eq. 2)}$$

where N-TAN(t=0) is the N-TAN concentration of the samples taken before the corresponding dosage of ammonium chloride and N-TAN(t=24) is the N-TAN concentration of the same taken 24 hours after. Ammonia removal rates, maximum and minimum concentrations are presented as mean ± standard deviation of their respective groups, minimum of four measurements (trial performed twice per two identical biofilters). A multifactorial ANOVA was performed to evaluate the significance of the three process parameters on the achievement of mean N-TAN removal rates. Multiple range tests (Tukey HSD) were performed to obtain significant differences on N-TAN removal rates depending on individual parameters and a multiple regression was performed to quantify the relationship between the parameters and the nitrification rate. Additionally, the mean maximum N-TAN concentration (among the values measured) value is indicated as well as the minimum N-TAN concentration (value of the 24th hour if ammonia remained in water), being these two factors relevant to fish welfare and survival. All ANOVAS and regressions were performed by Statgraphics® Centurion XVII for Microsoft Windows®.

3. Results

3.1. N-TAN, nitrite and nitrate hourly variation

Figure 4.2, Figure 4.3 and Figure 4.4 show the variation of N-TAN, $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ concentrations throughout the biofilter performance measurements, which were obtained every two hours for every combination of water temperature and hydraulic loading. N-TAN rapidly ascended after the TAN addition (which simulated fish feeding period) and in every case, rose until it reached a peak approximately after 6 hours, and then started to descend. The variation pattern was similar irrespective of hydraulic loading and temperature, although maximum and minimum N-TAN concentrations, as well as N-TAN decrease rate varied with each combination of process parameters. $N\text{-}NO_2^-$ showed a small variation for all trials, the concentration levels rose and decreased shortly after. The rise of NO_2^- concentration was notably bigger for the combination of water temperature of 27ºC and hydraulic loading of 11 and 8 $m^3\ m^{-2}\ h^{-1}$. $N\text{-}NO_3^-$ concentrations increased throughout the 24-hour period for every combination of temperature and hydraulic loading, the concentrations being dependent mainly on N-TAN concentrations. Nitrification was therefore achieved for every combination of process parameters.

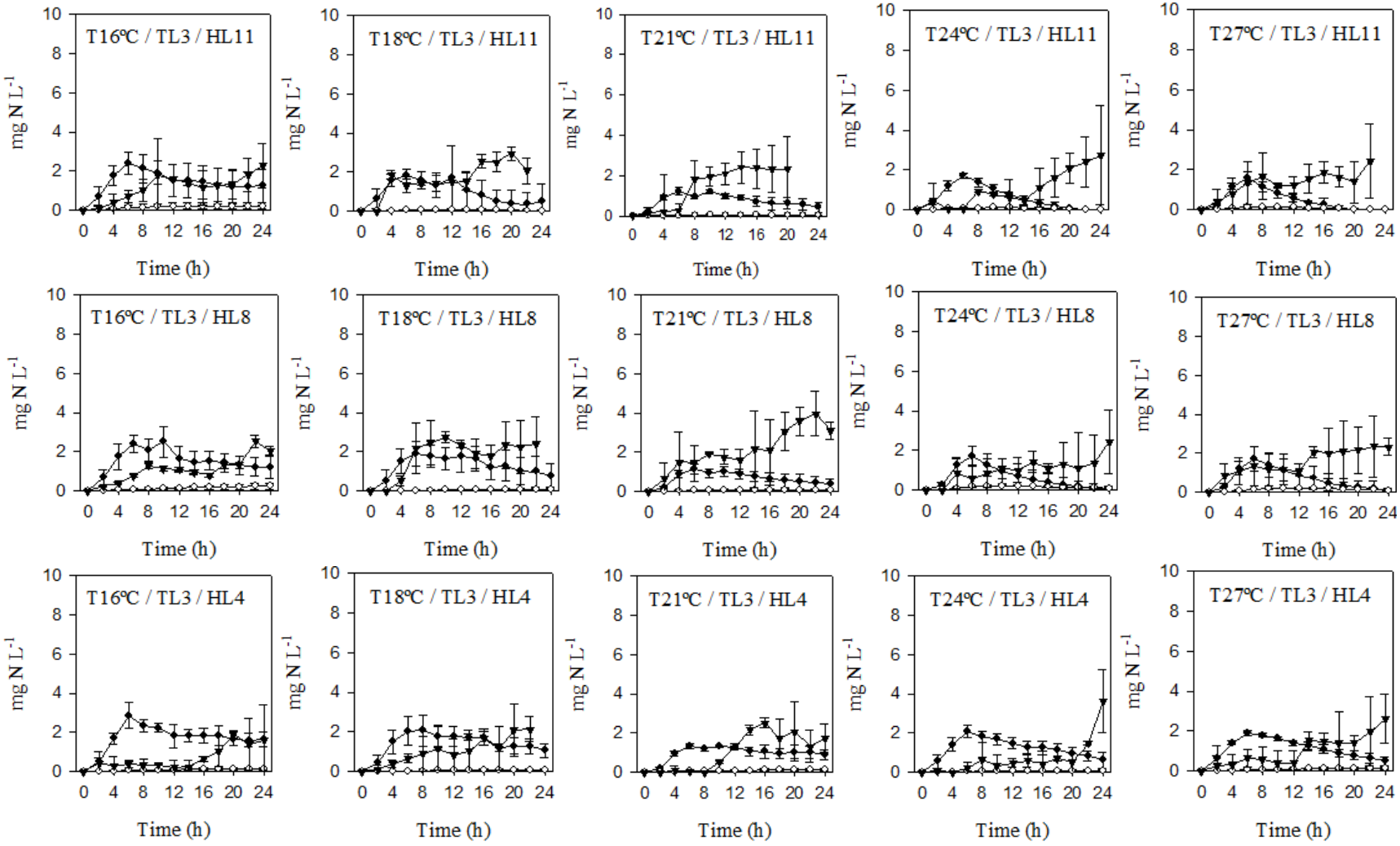

Figure 4.2. N-TAN (black circles), NO_2^- (white circles) and NO_3^- (inverted triangles) concentration variation for every combination of temperature and hydraulic loading at a TAN load of 3 gTAN m^{-3} day^{-1}

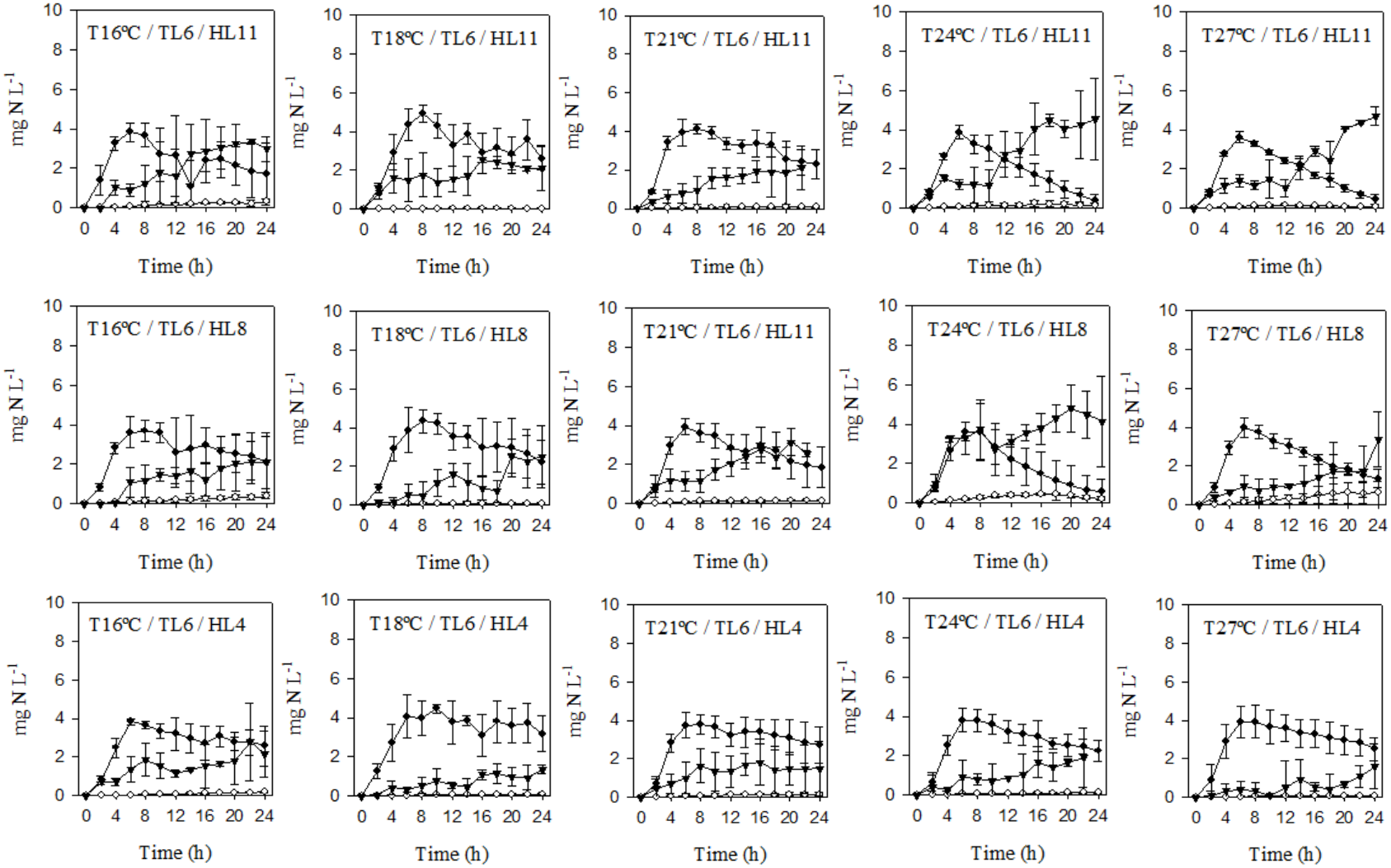

Figure 4.3. N-TAN (black circles), NO_2^- (white circles) and NO_3^- (inverted triangles) concentration variation for every combination of temperature and hydraulic loading at a TAN load of 6 gTAN m^{-3} day^{-1}

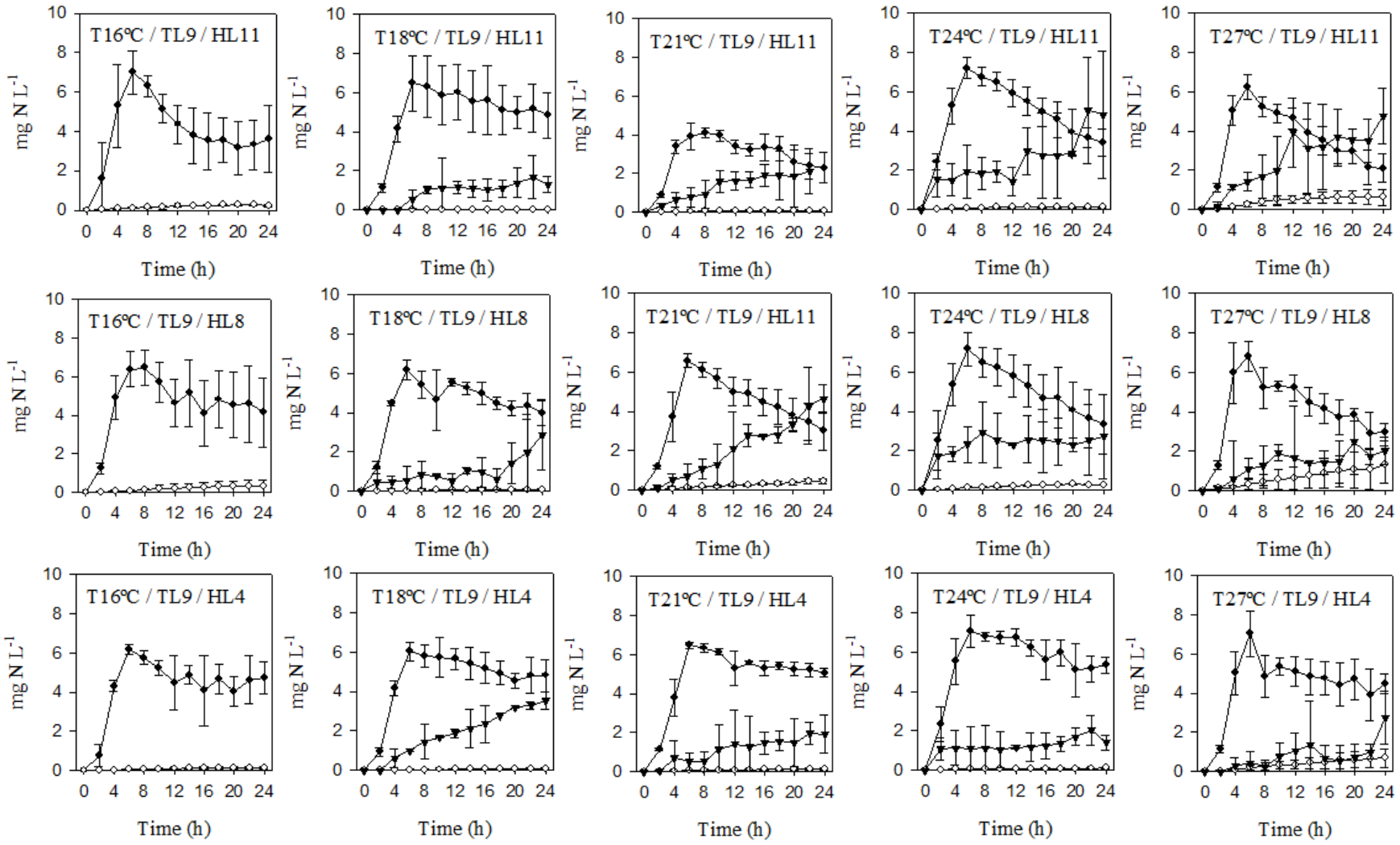

Figure 4.4. *N-TAN (black circles), NO_2^- (white circles) and NO_3^- (inverted triangles) concentration variation for every combination of temperature and hydraulic loading at a TAN load of 9 gTAN m^{-3} day^{-1}*

3.2. Alkalinity

Alkalinity (measured as $mgCaCO_3\ L^{-1}$) decreased unceasingly during the 24 hour trials. The reduction was directly related to the amount of TAN consumed by the bacteria as it can be seen in Figure 4.5. A linear regression is relatively well adjusted to the data (R=57.39%) although the random factor was quite high as well. Due to the constant replacement of tank water prior to biofilter performance measurements, alkalinity was similar for all tanks at time 0 (average=168.96 $mgCaCO_3\ L^{-1}$±1.72, relatively high due to the character of Valencian freshwater) and never was completely emptied after 24 hours (average=140.20 $mgCaCO_3\ L^{-1}$±4.55).

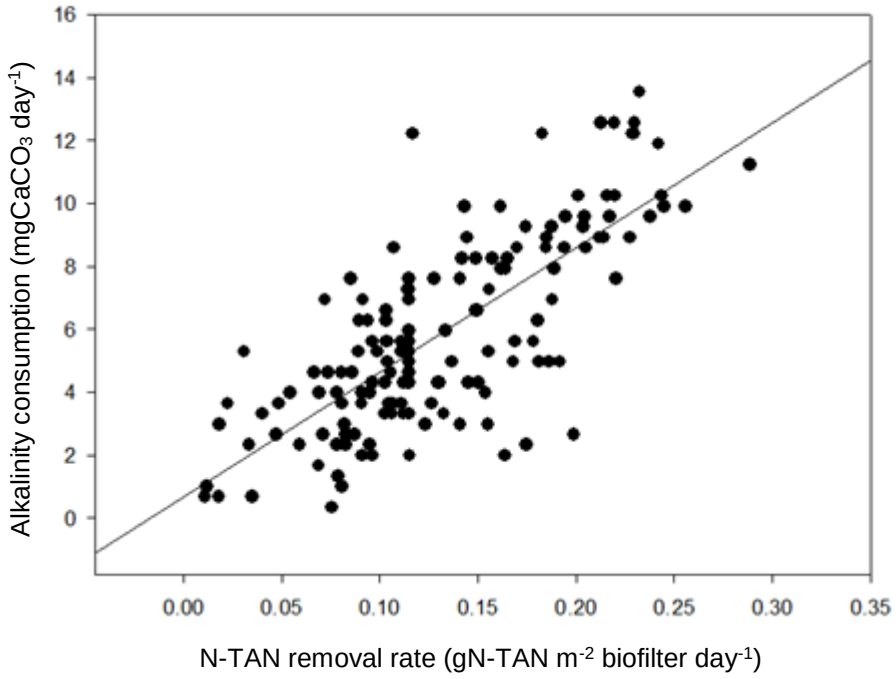

Figure 4.5. Scatter plot of the relationship between N-TAN removal rate and alkalinity consumption.

3.3. Influence of process parameters on N-TAN removal rate

Results of the multivariate ANOVA (Table 4.3) indicate that the three factors had a significant effect on the mean ammonia removal rate, as well as the interaction of TAN load with both temperature and hydraulic loading (p-value<0.05). Multiple range tests were made for each individual process parameter to analyze the sole influence of a particular process parameter on nitrification rates irrespective of the value of the remaining ones.

Table 4.3. Multivariate ANOVA table (α=0.05)

Parameters	***Sum of squares***	***Mean square***	***F-statistic***	***P-value***
Temperature	0.0832745	0.0208186	11.69	0.000
TAN load	0.141861	0.0709304	39.81	0.000
Hydraulic loading	0.0526804	0.0263402	14.78	0.000
Interactions				
Temperature w/ TAN load	0.0404043	0.00505054	2.83	0.0061
Temperature w/ Hydraulic loading	0.0167455	0.00209319	1.17	0.3188
TAN load w/ Hydraulic loading	0.0196251	0.00490628	2.75	0.0306
T w/ TAN load w/ Hydraulic loading	0.0215354	0.00134596	0.76	0.7325
Residues	0.240519	0.00178162		
TOTAL	0.616645			

In the case of temperature (Table 4.4), mean water temperatures of 24ºC and 27ºC led to the achievement of the highest mean N-TAN removal rates (0.146 and 0.157 gN-TAN m^{-2} day^{-1}, respectively), without significant differences between them. There were no significant differences of N-TAN removal rates between the remaining temperatures. On the other hand, there were no significant differences on maximum or minimum TAN concentrations observed at any temperature.

Table 4.4. Mean (+ standard deviation) N-TAN removal rates, maximum and minimum TAN concentrations depending on water temperature. Different superscripts in the same column indicate significant differences (p<0.05).

Temperature (ºC)	*n*	*gN-TAN removed m^{-2} day^{-1}*	*Maximum TAN concentration (mgN L^{-1})*	*Minimum TAN concentration (mgN L^{-1})*
16	36	0.107±0.012^{c}	4.407±0.300	2.607±0.280
18	36	0.107±0.007^{c}	4.357±0.326	2.734±0.300
21	36	0.127±0.009^{bc}	3.977±0.366	2.228±0.269
24	36	0.146±0.009^{ab}	4.426±0.397	2.573±0.473
27	36	0.157±0.008^{a}	4.191±0.373	2.052±0.302

Different superscripts in the same column indicate significant differences (p<0.05).

According to the hydraulic loading of the biofilters (Table 4.5), a hydraulic loading of 4 m^3 m^{-2} h^{-1} led to the lowest mean N-TAN removal rate. There were no significant differences between the mean N-TAN removal rate of biofilters whose hydraulic loading were 8 and 11 m^3 m^{-2} h^{-1} (0.137 and 0.143 gN-TAN m^{-2} day^{-1}). No significant differences were found on the maximum TAN concentrations for any hydraulic loading but mean minimum TAN concentration was found to be significantly higher (3.236±0.275 gN-TAN m^{-2} day^{-1}) for the hydraulic loading of 4 m^3 m^{-2} h^{-1}.

Table 4.5. N-TAN removal rates, maximum and minimum TAN concentrations depending on hydraulic loading.

Hydraulic loading (m^3 m^{-2} h^{-1})	*n*	*gN-TAN removed m^{-2} day^{-1}*	*Maximum TAN concentration (mgN L^{-1})*	*Minimum TAN concentration (mgN L^{-1})*
4	60	0.105±0.044[b]	4.478±2.121	3.236±2.133[a]
8	60	0.139±0.059[a]	4.166±2.118	2.144±1.869[b]
11	60	0.145±0.064[a]	4.170±2.102	1.936±1.745[b]

Different superscripts in the same column indicate significant differences ($p<0.05$).

The influence of TAN load proved to be also significant on nitrification rate (Table 4.6). Maximum mean N-TAN removal rates were obtained when 6 and 9 gTAN m^{-3} day^{-1} were added (0.141 and 0.154 gN-TAN m^{-2} day^{-1}, respectively). There were no significant differences between these two N-TAN removal rates. Maximum and minimum TAN concentrations augmented significantly with every increase of TAN load.

Table 4.6. N-TAN removal rates, maximum and minimum TAN concentrations depending on TAN load.

TAN load (gTAN m^{-3} day^{-1})	*n*	*gN-TAN removed m^{-2} day^{-1}*	*Maximum TAN concentration (mgN L^{-1})*	*Minimum TAN concentration (mgN L^{-1})*
3	60	0.092±0.032[b]	1.914±0.614[c]	0.714±0.631[c]
6	60	0.141±0.057[a]	4.141±0.620[b]	2.143±1.173[b]
9	60	0.154±0.062[a]	6.759±0.863[a]	4.459±1.744[a]

Different superscripts in the same column indicate significant differences ($p<0.05$).

A model generated with the equation of a multiple regression based on the data is presented in Figure 4.6. The influence of temperature and TAN load on nitrification rate was similar for every hydraulic loading tested, but the performance of biofilters with hydraulic loading of 4 m^3 m^{-2} h^{-1} showed considerably lower performance that the biofilters with other hydraulic loadings.

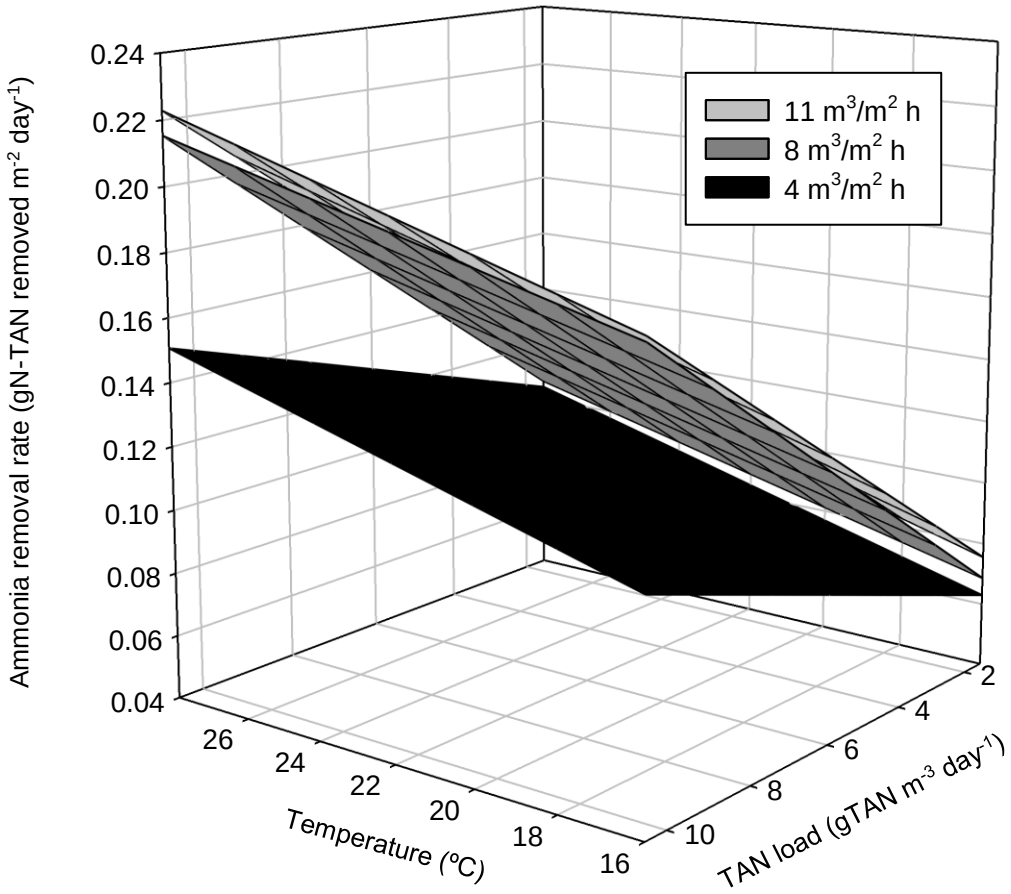

Figure 4.6. Multiple linear regression model plotting the relationship between ammonia removal rates depending on temperature and TAN load for every hydraulic loading tested

3.4. Influence of the combination of process parameters on N-TAN removal rates, maximum and minimum N-TAN concentrations

Differences on mean N-TAN removal rates, maximum and minimum N-TAN concentrations obtained on the trials in which process parameters' values were constant were analyzed with several one-way ANOVAS and multiple range tests (Tukey HSD). Results also provided information on the relative strength of the individual process parameters, based on the information gathered with the multivariate ANOVA as well as the one-way ANOVAS made for the singular parameters.

With regard to N-TAN removal rates (Table 4.7), it is observed that different hydraulic loadings at constant TAN loads and temperatures, were not particularly influential on nitrification rates at lower temperatures. However, as temperatures increased, hydraulic loading started to influence nitrification rates in the same matter as showed in Table 5, being the biofilters with hydraulic loading of 4 m^3 m^{-2} h^{-1} which significantly removed less ammonia from the rearing water. At higher temperatures (24ºC and 27ºC) and low TAN

load (3 gTAN m^{-3} day^{-1}) nitrification rates were very similar for every biofilter due to the entire amount of ammonia being removed from the rearing water.

Similarly, N-TAN removal rates generally increased along with the temperature, particularly on biofilters with a hydraulic loading of 11 m^3 m^{-2} h^{-1}.

Table 4.7. N-TAN removal rates for every combination of process parameters values (n=4).

Process parameters		***N-TAN removal rate (gN-TAN removed m^{-2} day^{-1})***				
TAN load (gTAN m^{-3} day^{-1})	*Hydraulic loading (m^3 m^{-2} h^{-1})*	*T=16ºC*	*T=18ºC*	*T=21ºC*	*T=24ºC*	*T=27ºC*
3	4	0.036±0.025^{B}	0.074±0.021^{AB}	0.087±0.016^{A}	0.106±0.024^{A}	0.113±0.014^{A}
	8	0.054±0.029^{B}	0.076±0.029^{AB}	0.097±0.012^{AB}	0.112±0.006^{A}	0.111±0.005^{A}
	11	0.054±0.042^{B}	0.091±0.045^{AB}	0.094±0.013^{AB}	0.115±0.000^{A}	0.115±0.000^{A}
6	4	0.121±0.065	0.080±0.058	0.113±0.059	0.143±0.032^{b}	0.126±0.037^{b}
	8	0.125±0.074	0.121±0.055	0.138±0.052	0.202±0.032^{a}	0.163±0.022^{b}
	11	0.144±0.077^{B}	0.101±0.029^{B}	0.117±0.038^{B}	0.211±0.017^{aA}	0.208±0.012^{aA}
9	4	0.123±0.051	0.117±0.051	0.102±0.014^{b}	0.079±0.027^{b}	0.140±0.031^{b}
	8	0.141±0.089	0.149±0.030	0.196±0.047^{a}	0.173±0.075^{a}	0.198±0.022^{ab}
	11	0.167±0.084^{AB}	0.106±0.056^{B}	0.202±0.011^{aA}	0.177±0.033^{aAB}	0.241±0.036^{aA}

Different superscripts in lowercase letters indicate significant differences ($p<0.05$) between hydraulic loadings (vertical) and different superscripts in capital letters indicate significant differences between temperatures (horizontal).

4. Discussion

Results of the multivariate ANOVA demonstrated that the three parameters studied (hydraulic loading, TAN load and water temperature) affect the performance of nitrifying trickling filters. A multiple linear regression was able to quantify well (R^2=0.88) the influence of the three process parameters within the ranges studied on biofilter performance, which cover a large range of temperatures and daily TAN loads. Figure 4.6 represents the range of possible nitrification rates based on the combination of process parameters. The efforts in the constant replacement of tank water as well as homogenization of other parameters critical to nitrification rates (such as $CaCO_3$ concentration) further reinforces the absence of interferences. Both initial and final alkalinity levels reached in the trails were apparently far from the levels required to reduce nitrification (Biesterfeld et al., 2003). Oxygen concentration was constant (6.499

± 0.025 mg L^{-1}) during the 24 hour measurements and did not become a limiting factor for any combination of variables. No significant drops of pH were observed as well (mean pH calculated was 8.363 ± 0.008).

In our experiment, TAN load had the most prominent influence on N-TAN removal rates. At the lowest TAN load (3 gTAN m^{-3} day^{-1}) mean N-TAN removal rate was significantly lower (0.092 gN-TAN m^{-2} day^{-1}) that at higher TAN loads, although there were no significant differences between the remaining TAN loads (6 gTAN m^{-3} day^{-1} and 9 gTAN m^{-3} day^{-1}) on nitrification rates. Usually it is also reported elsewhere (Díaz et al., 2012; van den Akker et al., 2008) a direct relationship between TAN load and nitrification rate up until a transition concentration, therefore our results are in concordance with literature. In these papers, lower biofilm capacity at low influent ammonia concentrations is usually explained by the lack of mass ammonia availability rather than diffusion limitations. In this paper, at lower TAN loads, all ammonia is removed from the bulk water provided temperature and hydraulic loading are favorable (Figure 4.2, Figure 4.3, Figure 4.4). N-TAN concentration reached 0 before the 24-hour period in some cases, which means that full biofiltration potential was not reached. However, increasing TAN load from 6 to 9 gTAN m^{-3} day^{-1} did not lead to an increasing nitrification rate which also shows as well the limitation of increasing ammonia influent concentration on improving biofilter performance. Nevertheless, N-TAN concentrations (both maximum and minimum), were significantly lower for the 6 gTAN m^{-3} day^{-1} TAN load, which may shift aquaculture management towards this particular process parameter value. They were as well significantly lower for the lowest TAN load tested (3 gTAN m^{-3} day^{-1}), therefore they might be a convenient tradeoff for achieving lower nitrification rates. As shown in Figure 4.5, final alkalinity concentrations were in average lower as well the bigger the nitrification rate was, although the decrease of pH through the trials were not significant. Therefore, no drawbacks are to be expected if $CaCO_3$ are kept to a minimum adding base to bulk water almost once a day (Summerfelt et al., 2015).

Biofilters with the lowest hydraulic loading tested (4 m^3 m^{-2} h^{-1}) showed a significantly lower performance compared with the biofilters with higher hydraulic loadings. This relationship between hydraulic loading and nitrification rate has been previously suggested by various researchers (Kamstra et al., 1998; Nijhof and Bovendeur, 1990), being the ammonia availability the basis of this relationship. They also stated that there is a limit on the positive influence of hydraulic loading on achieving higher TAN removal, which has been also demonstrated in this paper. Nitrification rates were not significantly higher for the highest hydraulic loading (11 m^3 m^{-2} h^{-1}) for any combination of TAN load

and temperature compared to the second highest (8 m^3 m^{-2} h^{-1}). Peng *et al*. (2003) tested similar hydraulic loadings used in our study, found comparable relationship between hydraulic loading rate and nitrification rates. They found no significant differences between nitrification rates for biofilter with 8.3 and 12.5 m^3 m^{-2} h^{-1}, but an increasing positive effect for lower hydraulic loading. Greiner and Timmons (1998) concluded that hydraulic loading did not show to be influential to nitrification rates, however the hydraulic loadings studied by them were far superior to the hydraulic loadings tested in this research (19.54 m^3 m^{-2} h^{-1} being their lowest and 11 m^3 m^{-2} h^{-1} being our highest). Literature suggests that insufficient wetting of the media is responsible for the reduced nitrification rates shown by low hydraulic loading biofilters. Van den Akker *et al.* (2008) investigated the effect of increasing water flow (thus increasing hydraulic loading) on the overall ammonia removal rate and concluded that biofilter performance was higher due to an active filtration overall biofilter area. Bacterial stratification inside biofilters has been observed by other authors (Sandu et al., 2002; van den Akker et al., 2008), and it would had possibly occurred in our experiment, due to the fact that the highest hydraulic loading biofilm was shaped as a tall cylinder in contrast to the second highest.

Hydraulic loading did not affect the maximum TAN concentration observed in the rearing water. However, significant differences were found in the minimum TAN concentration according to nitrification rate. Biofilters that showed higher nitrification rates removed more ammonia from the water and as a result after 24 hours the ammonia concentration was lower, but it did not prevent achieving similar peaks of TAN concentrations as with the other biofilters.

Temperature also affected nitrification rates. In particular, mean water temperatures of 24ºC and 27ºC led to the achievement of the highest mean N-TAN removal rates (0.146 and 0.157 gN-TAN m^{-2} day^{-1}, respectively). This direct relationship has been published on other articles (Lyssenko and Wheaton, 2006; Zhang et al., 2014), and according to the results found in literature as well as the results presented on this paper, there might be a biofilter performance increase at even higher temperatures, but these are going to be unlikely fit for the culture of marketable species. On the other hand, no significant differences were found on maximum or minimum N-TAN concentration between every temperature.

The combination of temperature and other process parameters had an interesting effect on biofilter performance (Table 4.7). For instance, only at high temperatures (21 ºC, 24 ºC and 27ºC) significant differences on N-TAN removal rates between hydraulic loadings

were observed. This suggests that if nitrifiers are inactivated by low temperature, their relative abundance and/or nitrification potential may not be perceived enough to establish a difference between several biofilter configurations. Considering only biofilters with a set hydraulic loading, it is also observed that different temperatures led to significantly different ammonia removal rates particularly at the lowest TAN load, as well as among the biofilters with hydraulic loading of 11 m^3 m^{-2} h^{-1} in the other TAN loads tested. The latter data may suggest a synergetic effect between temperature and hydraulic loading. The highest mean N-TAN removal rate (0.241 gN-TAN removed m^{-2} day^{-1}) was observed on the trials with biofilters with a hydraulic loading of 11 m^3 m^{-2} h^{-1}, water temperature of 27 ºC and TAN load of 9 gTAN m^{-3} day^{-1}.

In conclusion, this paper addresses the importance of the three process parameters studied, as well as their interactions with one another. Figure 4.6 as well as Table 4.7 provide a summary on the mean nitrification rates obtained by a large set of combinations of process parameters values expressed by N-TAN removed by day and biofiltration area. For the volume of the pilot scale biofilters used in this experiment, ammonia was not completely removed from the rearing water during the day for the majority of the process parameter combinations (Figure 4.2, Figure 4.3 and Figure 4.4), hence TAN is expected to be accumulated with every feeding. The quantity of ammonia removed from the bulk water could increase if larger biofilters (provided the hydraulic loading remains constant) are installed, although additional RAS engineering is perfectly suitable for improving N-TAN removal such as divide the effluent of the water tanks and pump it into two identical biofilters instead of one or even heating of the biofilter influent water or the biofilter itself with microwave heating as some authors have suggested (Zieliski et al., 2013), although probably not economically convenient.

Another important thing to consider is that operational hydraulic loadings are dependent on filter media (Eding et al., 2006), thus the nitrification rates obtained in the paper respective of hydraulic loading may be interesting for the design and construction of trickling filter with Bactoballs® (Aqua-Medic®, Bissendorf, Germany) or plastic filter media with similar characteristics.

CONCLUSIONS

TAN load, temperature and hydraulic loading proved to be influential on the achieving of particular ammonia removal rates, being the TAN load the most determinant. The relationship between TAN load and ammonia removal rate is, however, not linear, and increasing TAN load may only lead to increase the environmental ammonia in rearing water, harmful to fish welfare. Very low temperatures and hydraulic loadings inactivate biofilms and lead to this influence being much less noticeable.

CHAPTER 5

Influence of diet and feeding strategy on oxygen consumption and ammonia excretion of gilthead sea bream (*Sparus aurata*) raised in recirculating aquaculture systems and associated nitrifying trickling filter performance

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ABSTRACT

Gilthead sea bream (*Sparus aurata*) were raised in six individual recirculating aquaculture systems (RAS) whose biofilters' performance was analyzed based on the ammonia excretion profile derived from three different diets with assorted nutritional value (a control diet, a fish-meal based diet and a plant-meal based diet) and three feeding strategies (manual feeding to apparent satiation, automatic feeding with restricted ration and auto-demand feeding). The ammonia excretion patterns based on every combination of diet and feeding strategy, as well as ammonia removal rates (based in the total ammonia produced minus the ammonia in the tank water) are determined. Oxygen consumption rates were additionally determined. Mean oxygen consumption rates were dependent on mean weight (fish on early growth stages consumed the most whilst the consumption gradually decreased with growth) and no strong relationship between the combination of diet and feeding strategy and oxygen consumption rates were found. The control diet, with a higher protein content, led to the highest ammonia excretion over all (777.87 mgN-TAN kg biomass^{-1} day^{-1}), as well as higher ammonia removal rates across every feeding strategy (0.07, 0.10 and 0.17 gN-TAN removed m^{-2} biofiltration area day^{-1} for the manual, automatic and auto-demand feeding strategy, respectively). The ammonia removal rates were particularly high when fish were fed by auto-demand feeders and in combination with the low ammonia concentrations found in water throughout the trial makes this feeding strategy preferable for managing nitrogenous wastes in recirculating aquaculture systems.

1. Introduction

In recirculating aquaculture systems (RAS) ammonia is usually removed by nitrifying biofilters, adopted from wastewater treatment (Liao and Mayo, 1972). However, in contrast to wastewater, there is a considerably larger water flow to be treated with low ammonia concentration, which represents a challenge because biofilters are known to be less efficient (Piedrahita, 2003). However, these low concentrations are still toxic to fish (Colt, 2006), so complete elimination is aimed at. Another important factor is the variation of the ammonia concentration on the biofilter influent relative to the excretion patterns of the fish, heavily dependent on feeding (Dosdat et al., 1996; Echevarría et al., 1993; Leung et al., 1999), which also introduces an element of uncertainty when analyzing biofilter removal capabilities (Eding et al., 2006; Foss et al., 2009).

Because of this, the objective to improve biofilter performance for aquaculture has led to a series of meticulous studies. Several factors that affect nitrification performance including reactor-specific parameters (filter media, hydraulic loading) as well as water quality parameters such as temperature, influent ammonia concentration, pH or organic matter (Eding et al., 2006). Each one of these effects on nitrification rate have been investigated by several authors, with or without including live fish on their experiments (Godoy-Olmos et al., 2019, 2016; Zhu and Chen, 1999). Ammonia load is probably the process parameter that affects the most the ammonia removal rate. Several authors have stated ½-order substrate dependent kinetics (Díaz et al., 2012; Salvetti et al., 2006; von Ahnen et al., 2015) and whereas the quantification of the effects of other parameters have been reported as well, their effect is somewhat less influential (Lyssenko and Wheaton, 2006).

In real aquaculture farming situations, the total ammonia excretion is dependent on a vastly range of aquaculture practices such as biomass, feed intake (Leung et al., 1999), protein content of the diet (Ballestrazzi et al., 1994) and feeding regime (Wu and Gatlin, 2014). Studies on biofilter performance carried out using live fish (Gallego-Alarcón and García-Pulido, 2017; von Ahnen et al., 2015) usually use commercial diets and feeding strategies based on fixed rations. Very few studies use new diets, even though the rapid depletion of fish meal sources is bringing up the necessity of the inclusion of plant meal sources on fish diets (Martínez-Llorens et al., 2012; Monge-Ortiz et al., 2018, 2016). Moreover, there has been some research that has demonstrated the variation of ammonia excretion due to various degrees of fishmeal replacement on fish diet (Obirikorang et al., 2015). Besides changing ammonia excretion, experimental plant meal diets increase the amount of organic matter in the water, mainly due to lesser digestibility of nutrients (Davidson et al., 2016, 2013). Varying C/N ratio of biofilter influent has been proved to affect biofilter performance by modifying the equilibrium between nitrifiers and heterotrophs in favor of the later (Michaud et al., 2006; Zhu and Chen, 2001). Therefore, it should be interesting to conduct studies on biofilter performance using live fish fed with diets in which a percentage of fish meal is substituted by plant sources to fully comprehend the applicability of waste treatment technologies on future aquaculture systems.

Feeding strategy, the selected method for distribution of the feed ration to the farmed fish, is usually also put aside when addressing biofilter performance or even ammonia excretion. There currently exists a certain amount of different feeding strategies which

include the use of belt-feeders, automatic feeders/dispensers and ad libitum feeding systems, in which fish are able to activate them to release the food to the water at any time (Lekang, 2015). Manual feeding is also a viable feeding strategy if the fish farm is not too large and the farmer wants to visually inspect the eating behavior of the fish to optimize feed use, although the recurrence of feedings is limited to the availability of personnel. Automated feeders can be operated to distribute the food in several feeding periods. Nevertheless, only restricted feeding is possible by using these systems and there is no real control of the consumption of the feed by fish, as there is a natural variability in fish appetite (Lekang, 2015). This latter problem occurs also with demand feeders.

It follows from the above that the selection of a certain feeding strategy is a good indicator of feed intake and temporal distribution of the feed ration. Both factors lead to determinate ammonia variation curves on the bulk water, and therefore should be considered when evaluating biofilter performance. Since there is no general recommendation on fish feeding strategy and fish diet for maximizing biofilter performance, this paper aims at evaluate diurnal nitrogen variations based on the selection of three specific diets (one control diet and two experimental diets, a fish meal based one and a plant meal based one) and three feeding strategies (manual feeding, automatic feeding and auto-demand feeding) with the objective to determine differences on nitrification rates.

2. Material and methods

2.1. Experimental system design

Experiments were conducted on six independent recirculating aquaculture systems consisting in a fiberglass tank filled with 250 L of sea-water, an associated biofilter, a plastic net covered with sponges which served as a solid removal unit and a water pump (Oceanrunner® OR3500, Aqua-Medic®, Bissendorf, Germany). The six recirculating systems were present on the Laboratory of Aquaculture building in the Polytechnic University of Valencia. Biofilters were identical for every systems and consisted in a plastic cube filled with Bactoballs® (Aqua-Medic®, Bissendorf, Germany). Detailed characteristics of biofilters are presented in Table 5.1 and a simple diagram of the system is shown in Figure 5.1. An internal heater (EHEIM thermocontrol 300, Eheim GmbH, Deizisau, Germany) was added to the tanks to keep water temperature at 22 ºC.

Table 5.1. Characteristics of the trickling filters used in this study

Parameter	Unit	Value
Water flow	m^3/h	1.08
Diameter	cm	38
Cross sectional area	m^2	0.11
Height	cm	22
Volume	m^3	0.025
Filer media specific surface area	m^2/m^3	300
Biofilter area	m^2	7.5

On occasion the bulk water was partially replaced with fresh seawater that was stocked in a reserve water tank. Salinity was corrected accordingly by mixing stored seawater with dechlorinated freshwater. The sponges that formed the solid removal unit were cleaned once a week as well.

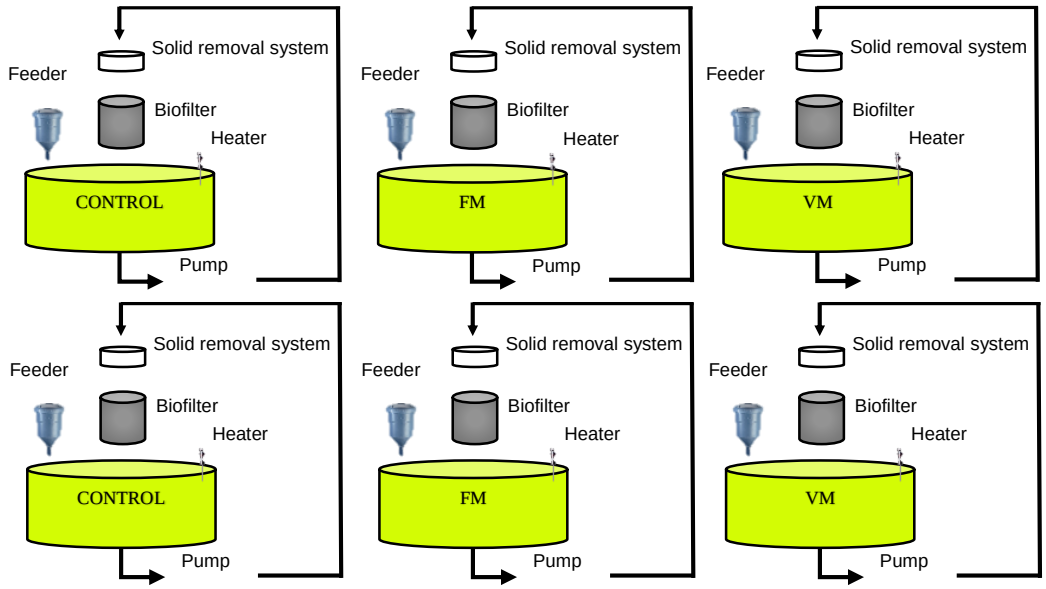

Figure 5.1. Diagram of the experimental systems. Feeders were not present when diets were supplied by hand.

2.2. Fish

A group of gilthead sea bream (*Sparus aurata*) (mean weight = 7.9 g at the beginning of the growth trial) were acclimated on the main recirculation system of the Laboratorio de Acuicultura (LAC) from the Universitat Politècnica de València, composed by 2000 L-filled cylindrical fiberglass tanks (18), with 75 m^3 of capacity total including a rotary mechanical filter and a 6m^3 capacity gravity biofilter. In that period fish were fed with the

control diet and after acclimation 10 fish were slaughtered and analyzed for proximate composition, serving as the initial fish for retention analyses. 30 of the remaining fish were placed in each tank and were subsequently fed with their assigned diet (one specific diet every two tanks) (Figure 5.1).

Feed were fed every day of the week except Sundays and feed intake was registered daily. Fish were regularly weighted every month after being anaesthetized with 10 mg/L clove oil (Guinama®), containing 87% eugenol. Fish used for retention analyses were slaughtered by a lethal dose of clove oil (150 mg/L). Five fish per tank were sacrificed during the first change in feeding strategy (57 days after the beginning of the trial) and three fish per tank were sacrificed during the second change in feeding strategy (109 days after the beginning of the trial). After every biofilter performance measurements were carried out, three fish per tank were slaughtered for the final proximate composition analysis. Of the remaining fish, five fish per tank were selected for the digestibility experiments.

2.3. Fish diets and feeding strategies

Three different diets were used in this experiment. One of the diets was a commercial diet which served as a control (CON) while the other two diets were mixed and pelleted using a semi-industrial twin-screw extruder (CLEXTRAL® BC-45, St. Etienne, France) at the feed producing laboratory of the Polytechnic University of Valencia. The processing conditions were: 0.63 g screw speed, 110 °C and 30–40 atm. Ingredients are shown in Table 5.2. The commercial diet was provided by a fingerlings producer (Blaumar S.A, Valencia, Spain), but the list of ingredients was not made available. However, it was analyzed alongside the experimental diets for carbon, nitrogen and fat content, as well as dry matter and ash content (values in Table 5.2).

Table 5.2. List of ingredients and proximate composition of the diets used in the study

Ingredients (g kg^{-1})	*Diets*	
	FM	**VM**
Fish meal	588.8	----
Wheat	260	----
Wheat gluten	----	295
Broad bean	----	41
Soybean meal	----	182
Pea meal	----	41
Sunflower seed meal	----	156
Soybean lecithin	10	10
Soybean oil	92.8	90
Fish oil	38.2	90
Calcium phosphate	----	38
Taurine	----	20
Methionine	----	7
Lysine	----	10
Arginine	----	5
Threonine	----	3
Vitamin mix	10	10

Proximate composition (% dry weight)	*Diets*		
	FM	**VM**	**CON**
Dry matter	88.1	93.9	93.6
Ash	10.1	7.4	10
Crude protein	44.2	45.0	59.0
Crude lipid	18.5	19.8	18.5
Digestible protein	42.7	41.8	48.6
Digestible energy (kJ g^{-1})	21.74	21.68	20.04
DP/DE (g kJ^{-1})	0.019	0.018	0.022

These diets were distributed by three different feeding strategies:

1. Manual feeding: feed was distributed to apparent satiation three times per day (9:00, 13:00 and 17:00). This was generally performed from Monday to Friday. On Saturdays, only one feeding was carried out. On Sundays fish were not fed.

2. Use of automatic feeders: feed was distributed using aquarium disc-feeders filled every day with a fixed ration based on fish biomass. Feeders were operated to distribute a portion of the ration 4 times a day (10:00, 13:00, 16:00 and 19:00). The following day the remainder of the daily meal (if any) was removed and weighted to calculate the feed intake before filling it once more. On Sundays the automatic feeders were not filled.

3. Use of auto-demand feeders (Ad libitum): a weighted portion of fish feed for each tank was placed on the hopper of auto-demand feeders, superior to the estimated feed intake to avoid shortage of feed. Fish were encouraged to activate a mechanism for a certain quantity of feed to drop on the water. The following day, the remainder of the daily meal was weighted and subtracted from the meal inserted the day before for the calculation of the feed intake before filling it once again. On Saturdays a greater portion of diet was placed on the hoppers to provide meal until the following Monday.

These three feeding strategies were adopted consecutively. Fish was distributed by hand until the completion of three measurements of ammonia excretion, oxygen consumption and biofilter performance for each tank. Fish were fed by automatic feeders afterwards until three new measurements of ammonia excretion, oxygen consumptions and biofilter performance were executed and fish were fed by auto-demand feeders afterwards. In total 54 trials were performed for every target parameter (ammonia excretion, oxygen consumption, ammonia removal rate)

2.4. Digestibility experiment

Digestibility experiment was carried out in the main recirculating aquaculture system of the Laboratory of Aquaculture (LAC) of the Universitat Politècnica de València. Fish were fed with a special variation of their respective feed including an inert market (chromic oxide) and faeces were collected every day by stripping. Nitrogen content of feed and the faeces as well as concentration of the inert marker of feed and faeces were analyzed

to calculate the apparent protein digestibility coefficient (APDC, %) by the following equation:

$$APDC(\%) = 100\ x\ (1 - \left(\frac{F}{D} \times \frac{DCr}{FCr}\right) \quad \text{(Eq. 3)}$$

where F is the percentage of nutrient in faeces, D is the percentage of nutrient in the diet, DCr is the percentage of chromic oxide in the diet and FCr is the percentage of chromic oxide in the faeces (Cho and Kaushik, 1990).

2.5. Nitrogen retention calculations

The amount of nitrogen retained by fish based of the amount of protein offered was calculated for every diet (Eq. 4). Protein in fish (whole-body) was analyzed for every change in feeding strategy (4 times total if initial fish were considered). These observations, coupled with the ammonia excretion measurements are performed with the objective of elaborating a nitrogen mass-balance similar to those described in Dalsgaard and Pedersen (2011) and Morales *et al.* (2018).

$$Retained\ protein\ (\%) = \frac{(\%\ Protein\ in\ fish_{t1} \times Biomass\ (g)_{t1}) - (\%\ Protein\ in\ fish_{t0} \times Biomass\ (g)_{t0})}{Feed\ intake_{t1-t0} \times \%\ Protein\ in\ diet} \quad \text{(Eq. 4)}$$

2.6. Ammonia excretion and oxygen consumption trails

Ammonia excretion and oxygen consumption were estimated based on the difference on ammonia/dissolved oxygen concentration on a given time. To ensure that no ammonia was eliminated by the biofilter during that time, the activity of the biofilter was cut out by switching off the pump that carried the water from the tank to the biofilter. On the same manner, the aeration system was turned off. Thus, tanks remained isolated 30 minutes every two hours, in which ammonia could only accumulate by fish excretion and oxygen only decrease by fish consumption, until the 24th hour on the six pilot-scale recirculating systems. Feeding was carried out as usual during the ammonia excretion/oxygen consumption trails.

Ammonia excretion and oxygen consumption were calculated in each given time using the following equations (Eq. 5 and Eq. 6, respectively) and data was used to estimate mean ammonia excretion/oxygen consumption in an hourly and daily basis. Results were

standardized in two ways: oxygen consumption/ammonia excretion by kg fish and oxygen consumption/ammonia excretion by kg fish and feed intake, dividing ΔDissolved oxygen/ammonia mass by fish biomass and feed intake, due to the high variance of these two parameters alongside the experiment and the relationship with the amount of depleted oxygen and ammonia excreted. As both ammonia excretion and oxygen consumption decrease with increasing body weight (Cai and Summerfelt, 1992) and the experiment was designed to change feeding strategies alongside the fish growth, mean weight was considered a co-variable when analyzing the effects of feeding strategy on ammonia excretion and oxygen consumption.

$$Ammonia\ excretion\ \left(\frac{mg}{h}\right) = \frac{Final\ N{-}TAN\ mass\ (mg)_{t0} - Initial\ N{-}TAN\ mass\ (mg)_{t1}}{time\ (h)_{t1}} \quad \text{(Eq. 5)}$$

$$Oxygen\ consumption\ \left(\frac{mg}{h}\right) = \frac{Initial\ O_2\ mass\ (mg)_{t0} - Final\ O_2\ mass\ (mg)_{t1}}{time\ (h)_{t1}} \quad \text{(Eq. 6)}$$

2.7. Biofilter performance measurements

Biofilter performance is calculated and presented as total ammonia nitrogen (N-TAN) removed per biofiltration area and day. N-TAN removal is deduced according to the following equation (Eq. 7) by difference between initial and final N-TAN in the bulk water assuming a determinate N-TAN excretion during that given period of time.

$$Ammonia\ removal\ rate\ \left(\frac{g\ N{-}TAN}{m2\ day}\right) = \frac{Initial\ N\text{-}TAN\ mass\ (t0) + Excreted\ N\text{-}TAN\ mass\ (t0\ to\ t1) - Final\ N\text{-}TAN\ mass\ (t1)}{biofiltration\ area \times time\ (t1)} \quad \text{(Eq. 7)}$$

The ammonia excretion rate estimates were taken from the ammonia excretion trials performed with the same fish (similar weight) on the same tanks fed by the same diet and feeding strategy. An average of the TAN excretion values obtained through the three replicate trials in the same timeframe was used for these calculations. However, the exact amount of ammonia excreted used for the calculation of ammonia removal rates were adjusted considering the feed intake that occurred on the day of the performance calculations, as well as the actual biomass of the tanks in the occurrence of a sudden casualty.

N-TAN concentrations for the N-TAN removal rates were determined every two hours for 24 hours, thus coinciding with the same fractions of time as the ammonia excretion measurements.

At the same time that samples were taken for N-TAN measurements, pH, temperature, dissolved oxygen, nitrite and nitrate were also analyzed.

2.8. Analytical procedures

2.8.1. Water quality parameters measurements

Temperature and dissolved oxygen (where applicable) was measured directly on the tank water by a Handy Polaris® oximeter (OxyGuard®, Farum, Denmark). pH was also measured directly on the water by a pHmeter of the same brand (Handy Polaris® pH, OxyGuard®, Farum, Denmark). For the measurement of TAN, NO_2^-, and NO_3^- a 50 mL sample was taken and analyzed at the laboratory shortly after collection by spectrophotometry.

TAN was measured by the indophenol method (Solorzano, 1969) after the addition of phenol, nitroprussiate, sodium citrate and DTT. After 6 hours, the absorbance of the compound was measured at a wavelength equal to 640 nm. Nitrite was measured by the Griess determination (Griess, 1879), after addition of sulfanilamide and N-(1-naphthyl)ethylendiamine dihydrochloride, which generates a pink/red dye whose absorbance (measured at 540 nm after 10 min) is proportional as well to nitrite concentration. For the measuring of nitrate, a novel determination was carried out based on the use of vanadium(III) chloride as a reducing agent, first described in the paper of Miranda, Espey and Wink (2001) and developed by Schnetger and Lehners (2014) for microplate readers. Measurements of nitrate were carried out after approximately 24 hours of reaction at room temperature. TAN and nitrite were measured using a T60V UV-VIS spectrophotometer (PG Instruments, Leicester, UK), while nitrate determinations were performed with a Victor 1420 microplate reader (Perkin Elmer, formerly Wallac Oy, Massachusetts, USA).

2.8.2. Fish and diets proximate composition

Proximate composition of the experimental diets and whole-body fish were determined according to the following procedures, after homogenization with a Waring® lab bender (Conair Corporation, One Cummings Point Road, Stamford, U.S.A): moisture was determined by oven thermal drying at 105°C to constant weight, ash by combustion in a muffle at 550°C overnight. Carbon and nitrogen were analyzed by the Dumas principle (TruSpec CN; Leco Corporation, St. Joseph, MI, USA), and used to determine protein content and energy. Energy was calculated according to Brouwer (1965), from the C (g) and N (g) balance (GE = 51.8 × C + 19.4 × N). Crude lipid was determined by diethyl ether extraction using an ANKOM XT10 Extractor.

2.8.3. Digestibility

Chromic oxide (the inert marker used for the digestibility experiments) was determined in the diets and in the faeces collected from the fish by stripping after acidic digestion with HNO_3 1.5 N + KCl 0.38%. The chromic oxide (VI) produced by the acidic digestion was measured by molecular spectrophotometry after the reaction with 1,5-diphenylcarbazide, which produces a red/violet compound whose absorbance (550 nm) is dependent of the chromic oxide concentration. This procedure was performed using the paper of Bremer Neto *et al.* (2005) as a reference.

2.9. Data processing and statistics

pH, dissolved oxygen and temperatures values are presented as given by the respective apparatus. TAN, nitrite and nitrate were measured as dissolved nitrogen concentration ($mgN\ L^{-1}$) and transformed to mass units (mgN) for calculation of ammonia excretion and ammonia removal.

Data of ammonia excretion are presented as ammonia nitrogen produced (mgN-TAN) divided by fish biomass (kg) and time (h). Oxygen consumption is presented as dissolved oxygen consumed (mgO_2) divided by fish biomass (kg) and time (h). Two series of data are presented depending on the focus of the trials. On one hand different oxygen consumption and ammonia excretion values happening depending on the hour of the

day are plotted with the objective of discussing daily variation between every combination of diet and feeding strategy. On the other hand, all the individually measured oxygen consumption/ammonia excretion values during one day where pooled to analyze significant differences between treatments. Ammonia removal rates are presented as g of N-TAN removed divided by biofiltration area (identical for every tank) and day, and were pooled from every ammonia removal rate calculated every two hours.

One-way and multivariate ANOVAS were performed to determine significant differences between single and combined treatments, respectively. All statistical analyses were carried out using Statgraphics® Centurion v.XVII.II for Windows®

3. Results

3.1. Growth, survival and proximate composition

Growth (Figure 5.2) was constant but uneven among the fish groups, in correspondence with the quality of the fish feed. The protein-rich commercial diet led to the highest mean weight of the fish (Table 5.3) being the experimental fish meal diet the second best feed for growth. Differences in growth between those groups (control and experimental fish meal diet) were practically non-existent up until the 82th day of the trial, when differences in mean weight started to become more apparent. The plant meal based diet started to impair not only growth but also survival very early on the study.

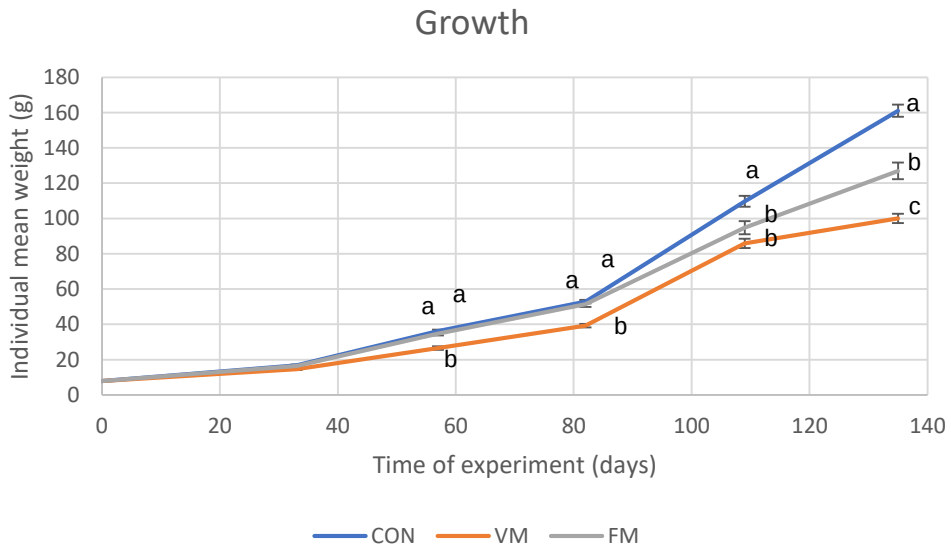

Figure 5.2. Fish growth (expressed in mean weight (g)) based on fish diet. Error bars (both positive and negative) indicate standard errors of the mean. Different letters indicate significant differences in individual main weight.

Table 5.3. Performance of gilthead sea bream fed experimental diets throughout the study.

	Experimental diets					
	CONTROL	***FM***	***VM***	***Mean***	***SEM***	***p-value***
Initial weight (g)	9.7	9.7	9.7			
Final weight (g)	161^{a}	127^{ab}	100^{b}	129	8.2	0.0307
Survival (%)	98.3^{a}	88.3^{ab}	76.7^{b}	87.8	3.04	0.0343
SGR (%/day)	2.23^{a}	2.05^{ab}	1.88^{b}	2.05	0.05	0.0355
Feed intake (g/100g fish)	1.51^{c}	1.67^{b}	2.06^{a}	1.75	0.26	0.0014
Feed conversion ratio	1.17^{c}	1.35^{b}	1.80^{a}	1.44	0.03	0.0015

Different superscripts in the same row indicate significant differences ($p<0.05$).

3.2. Oxygen consumption

As shown in Figure 5.3, the variation patterns in oxygen consumption were similar for every diet supplied (not in amount of oxygen consumed by rather in increases and decreases) irrespective of feeding strategy, although there were quite different between feeding strategies. After a constant rise that stopped after 6 hours approximately, oxygen consumption rate was somewhat constant in the trial in which feed was given *ad libitum* to the fish, whereas the variations in hourly oxygen consumption were more abrupt with the manual and automatic feeding strategy. The exact moments were feed was given are pointed out in the graph, showing a possible relationship between feeding and oxygen consumption rate.

Table 5.4. Mean oxygen consumption rates depending on feeding diet.

Diet	*Oxygen consumption*	
	gO_2 kg biomass^{-1} day^{-1}	***mgO_2 kg biomass^{-1} g feed intake^{-1} day^{-1}***
CON	12.48 ± 0.77^{b}	349.44 ± 39.30^{b}
FM	12.79 ± 0.95^{b}	370.24 ± 41.11^{b}
VM	15.31 ± 1.52^{a}	460.15 ± 44.25^{a}

Different superscripts in the same column indicate significant differences (p value<0.05).

There were significant differences in oxygen consumption based on diet (Table 5.4). Fish belonging to the VM group presented a higher significant oxygen consumption rate, including and not including consumed feed into account (460.15±44.25 mgO_2 consumed

kg biomass^{-1} g feed intake^{-1} day^{-1} and 15.31±1.52 gO2 consumed kg biomass^{-1} day^{-1}, respectively). No significant differences were found between the remaining diets.

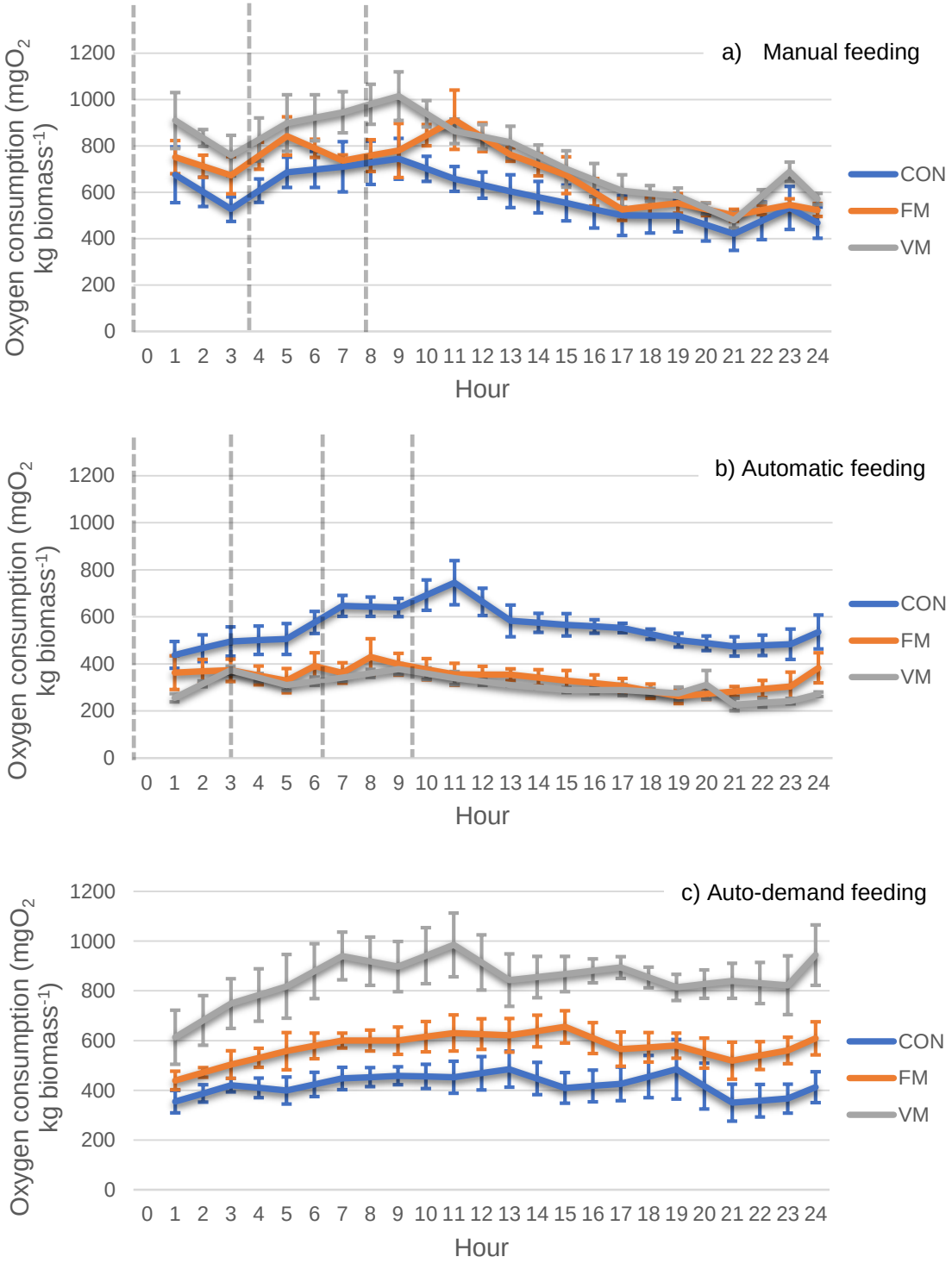

Figure 5.3. Oxygen consumption rates' hourly variation selected by feed for every feeding strategy tested: (a) manual feeding, (b) auto-feeders, (c) auto-demand feeders. Error bars (both positive and negative) indicate standard errors of the mean. Dashed lines indicate periods of feeding when applicable.

Feeding strategy was also proven to be significantly influent (p-values=0.0049 and 0.0319 for rates without including feed intake and including feed intake, respectively) on oxygen consumption (Figure 5.4 and Figure 5.5). Although the manual feeding strategy led to the highest mean oxygen consumption rate over all (16.04±0.69 gO_2 consumed kg biomass^{-1} day^{-1} and 511.73±34.30 mgO_2 consumed kg biomass^{-1} g feed intake^{-1} day^{-1}), there were differences in oxygen consumption between the remaining feeding strategies depending on the consideration of feed intake. Fish fed by auto-demand feeders showed an oxygen consumption not significantly different from the manual fed fish if feed intake was not considered. However, it was the automatic feeding strategy which led to the second highest mean consumption rate when feed intake was considered. Nevertheless, a separate ANCOVA analysis proved the mean weight to be a significant co-variable for the oxygen consumption rate results, both including and not including feed intake as a part of the oxygen consumption rate (p-values=0.0162 and 0.0026), which will be considered when discussing the results. No other variables did have a significant effect (p-value>0.05) when considering them as co-variables.

The influence of every combination of factors on the mean oxygen consumption rate, is represented as well in Figure 5.4 and Figure 5.5. Fish whose oxygen consumption was the highest (20.06±1.80 gO_2 consumed kg biomass^{-1} day^{-1}) were in the group fed with the VM diet with auto-demand feeders.

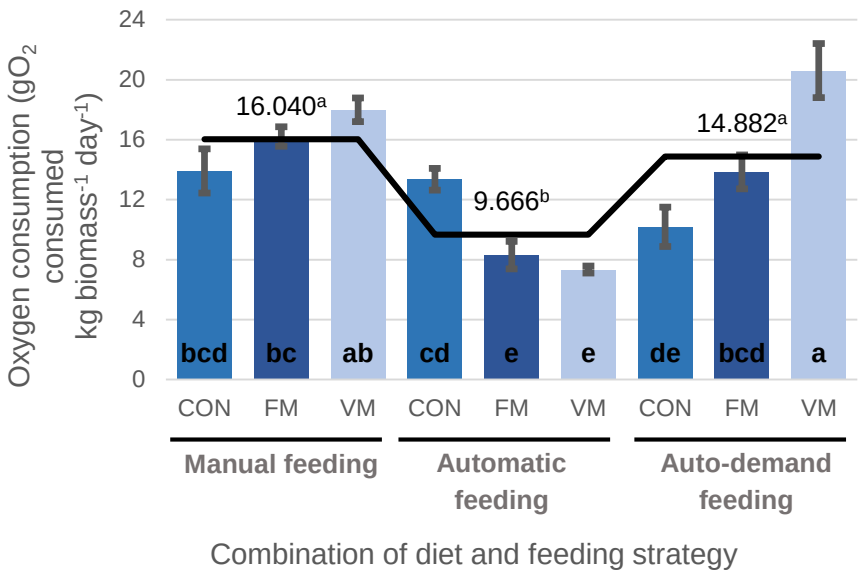

Figure 5.4. Mean oxygen consumption (n=6) for every combination of diet and feeding strategy, not considering feed intake. Error bars represent standard errors of the mean. For the bars, different letters in tags indicate significant differences (p-value<0.05). The solid line represents mean oxygen consumption rates for every feeding strategy alone. Letters in superscript represent significant differences between means (p-value<0.05).

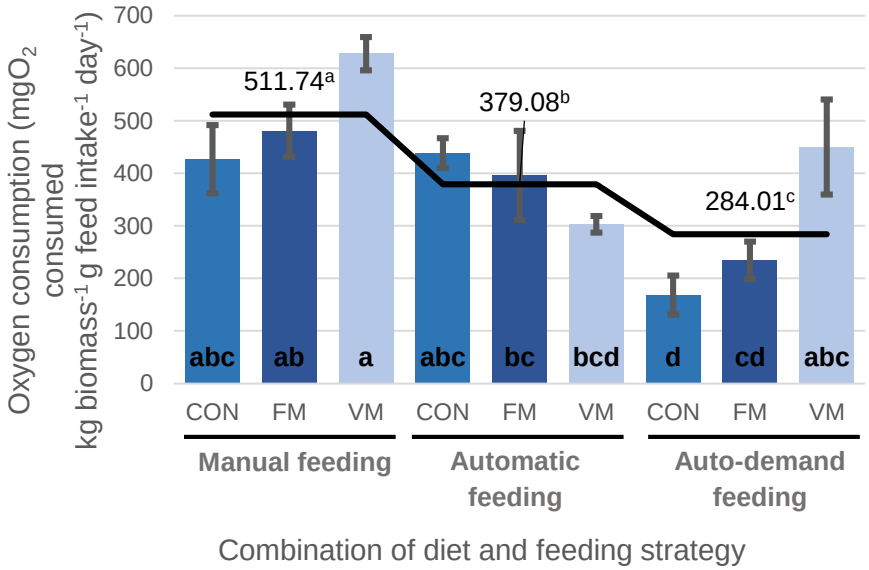

Figure 5.5. Mean oxygen consumption (n=6) for every combination of diet and feeding strategy, considering feed intake. Error bars represent standard errors of the mean. Different letters in tags indicate significant differences (p-value<0.05). The solid line represents mean oxygen consumption rates for every feeding strategy alone. Letters in superscript represent significant differences between means (p-value<0.05).

3.3. Ammonia excretion

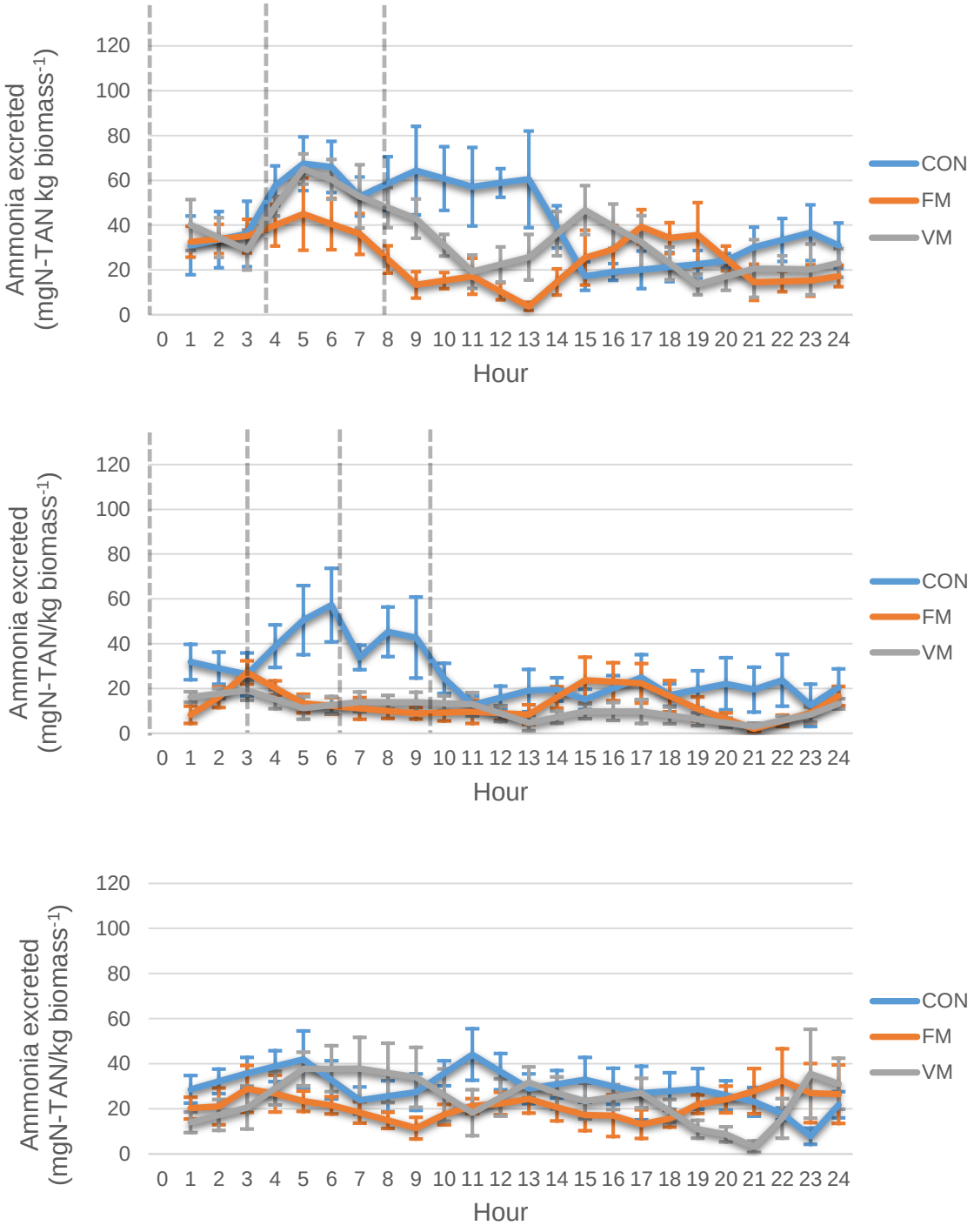

Figure 5.6. Ammonia excretion rates' hourly variation selected by feed for every feeding strategy tested: (a) manual feeding, (b) auto-feeders, (c) auto-demand feeders. Error bars (both positive and negative) indicate standard errors of the mean. Dashed lines indicate periods of feeding when applicable.

Figure 5.6 represents the variation of ammonia excreted throughout the day. Sudden raises in ammonia excretion followed manual feedings, more acutely pronounced that in the case of oxygen consumption rates, particularly for fish fed the control diet. When fish were fed with feeders (either automatic or auto-demand) the ammonia excretion was particularly low and more constant throughout the day.

A multivariate ANOVA analysis demonstrated the two effects (diet and feeding strategy), as well to be significantly influential in ammonia excretion rates by fish biomass and feed intake (p-values=0.0021 and 0.0000 respectively) as well as in ammonia excretion rates by fish biomass alone (p-values=0.0002 and 0.0000, respectively). Differences between diets are represented in Table 5.5 while differences between feeding strategies and excretion based on the combination of diet and feeding strategy are represented in Figure 5.7 and Figure 5.8.

Table 5.5. Mean ammonia excretion rates depending on the different diets fed.

Diet	*Ammonia excretion*	
	mgN-TAN kg biomass^{-1} day^{-1}	***mgN-TAN kg biomass^{-1} g feed intake^{-1} day^{-1}***
CONTROL	777.87±56.67^{a}	20.13±1.91^{a}
FM	481.46±45.78^{b}	13.52±1.47^{b}
VM	609.87±93.36^{b}	18.65±2.97^{a}

Different superscripts in the same column indicate significant differences (p value<0.05).

Fish fed the control diet excreted the most amount of N-TAN per kg (777.87±56.67 mg) with no significant differences among the remaining diets. On the other hand, both the control diet and the VM diets excreted significantly higher daily ammonia per g of feed intake (20.13±1.91 mgN-TAN and 18.65±2.97 mgN-TAN, respectively) than fish fed the FM diet. Considering feeding strategies, fish fed via manual feeding excreted the most daily ammonia on average, either per kg of fish and per kg of fish and g of feed intake. Concretely, a mean excretion of 800.35±58.57 mgN-TAN kg biomass^{-1} day^{-1} and 26.52±2.21 mgN-TAN kg biomass^{-1} g feed intake^{-1} day^{-1} was observed. Mean ammonia excretion rate via automatic feeding was significantly higher (15.30±1.54 mgN-TAN kg biomass^{-1} g feed intake^{-1} day^{-1}) than mean ammonia excretion rate when fish were fed by auto-demand feeders (10.48±0.91 mgN-TAN kg biomass^{-1} g feed intake^{-1} day^{-1}), considering feed intake. Nevertheless, while considering ammonia excretion per kg of fish, it was higher for the fish fed via auto-demand feeders (614.34±60.93 mgN-TAN kg biomass^{-1} day^{-1}) than for the fish fed via automatic feeders (405.61±49.48 mgN-TAN kg

biomass^{-1} day^{-1}). Contrarily to oxygen excretion, mean weight did not have an effect on ammonia excretion (p-value>0.05). Neither did any other possible co-variables.

When taking all combination of factors into consideration, fish whose ammonia excretion was the biggest (983.28±92.52 mgN-TAN kg biomass^{-1} day^{-1}) were in the group fed with the CON diet to satiation (manual feeding).

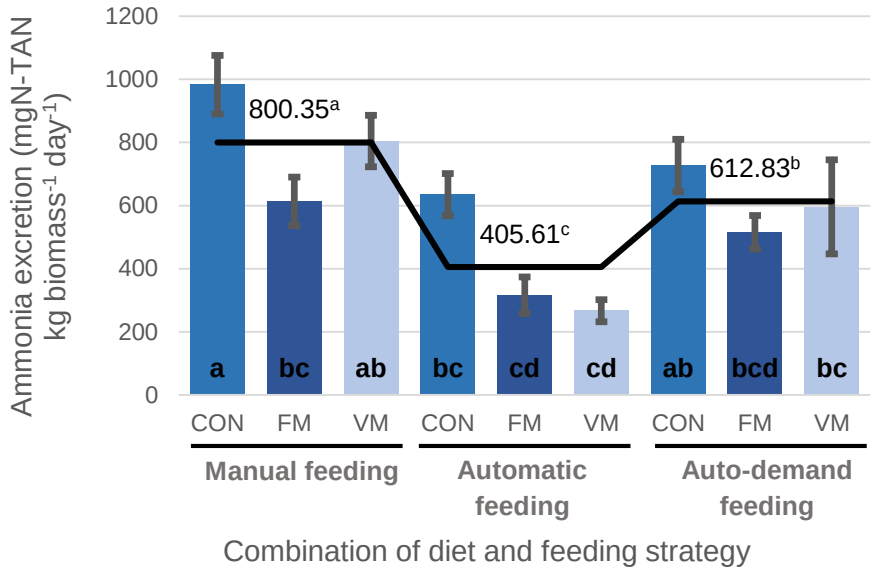

Figure 5.7. Mean ammonia excretion rate (n=6) for every combination of diet and feeding strategy, not considering feed intake. Error bars represent standard errors of the mean. For the bars, different letters in tags indicate significant differences (p-value<0.05). The solid line represents mean oxygen consumption rates for every feeding strategy alone. Letters in superscript represent significant differences between means (p-value<0.05).

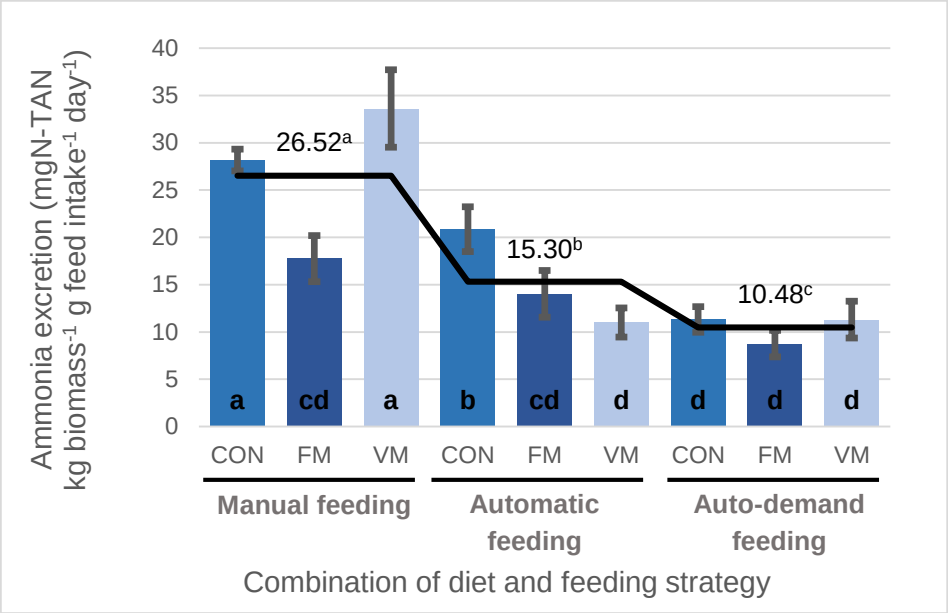

Figure 5.8. Mean ammonia excretion rate (n=6) for every combination of diet and feeding strategy, considering feed intake. Error bars represent standard errors of the mean. For the bars, different letters in tags indicate significant differences (p-value<0.05). The solid line represents mean oxygen consumption rates for every feeding strategy alone. Letters in superscript represent significant differences between means (p-value<0.05).

3.4. Nitrogen balance

Nitrogen retention, ammonia excretion rates and solid nitrogenous waste (calculated as 100 - ADC of the nitrogen for every diet) are represented in a nitrogen mass-balance (Figure 5.9). There were differences between diets for every one of the nitrogenous waste fractions. Nitrogen retention was about 30% for every diet, and there were significant unaccounted sources of nitrogen, especially in the case of the experimental diets (FM and VM).

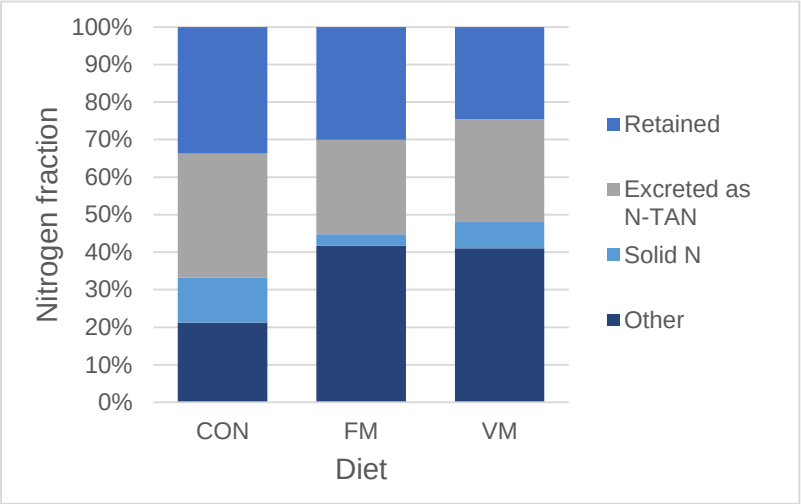

Figure 5.9. Nitrogen fraction allocation of dietary protein intake for every experimental diet.

3.5. Biofilter performance

Biofilters in trials with fish fed the control diet removed the most ammonia from water (0.112±0.009 gN-TAN removed m^{-2} biofiltration area day^{-1}). There were no significant differences between the remaining experimental groups (Table 5.6). The use of auto-demand feeders led to the significantly greater mean ammonia removal rate by the biofilters, with no significant differences between the remaining feeding strategies (Table 5.7).

Table 5.6. Ammonia removal rates depending on the different diets fed.

Diet	*Ammonia removal rate (gN-TAN removed m^{-2} biofiltration area day^{-1})*
CONTROL	0.11 ± 0.01^{a}
FM	0.06 ± 0.01^{b}
VM	0.05 ± 0.01^{b}

Different superscripts indicate significant differences (p value<0.05).

Table 5.7. Ammonia removal rates depending on the different feeding strategies.

Feeding strategy	*Ammonia removal rate (gN-TAN removed m^{-2} biofiltration area day^{-1})*
Manual	0.05 ± 0.01^{b}
Automatic	0.07 ± 0.01^{b}
Auto-demand	0.10 ± 0.01^{a}

Different superscripts indicate significant differences (p value<0.05).

The effect of the combination of diet and feeding strategy is represented in Figure 5.10, as well as the maximum ammonia found on the rearing one among the samples that were measured every two hours. Every concentration, as well as the measurements of nitrite and nitrate are shown in Figure 5.11 for every combination of diet and feeding strategy. Any amount of N-TAN that could have been present at the beginning of the trials was standardized to zero and was subtracted from each sample to facilitate comprehension.

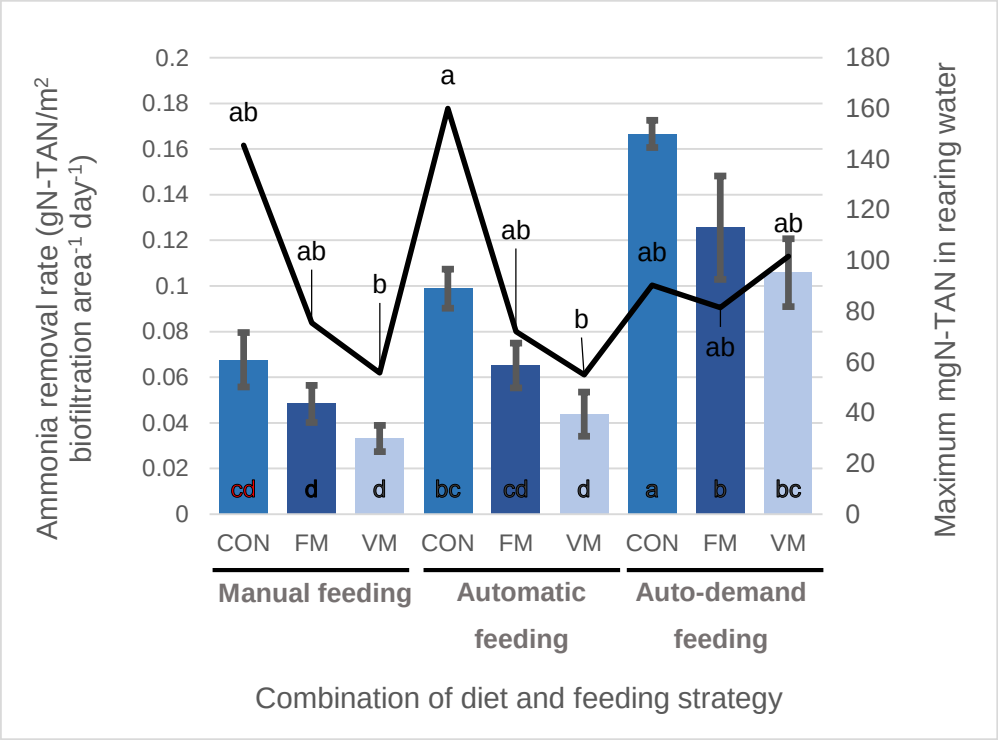

Figure 5.10. Effect of the combination of diet and feeding strategy on ammonia removal rates (bars) and maximum ammonia (mgN-TAN) found of the rearing water in the trial day. Different letters (next to the horizontal axis for the ammonia removal rates and in the proximity of the data points for the maximum N-TAN) indicate significant differences (p-value<0.05).

Ammonia removal rates were particularly high when feed was distributed by the use of auto-demand feeders, and the use of the control diet led to generally higher removal among every feeding strategy. In the case of the manual feeding as well as in the case of the automatic feeding strategy there was a close relationship between the maximum N-TAN found in water and the ammonia removal rate, nevertheless in the case of the auto-demand feeders, there were no differences in maximum N-TAN although there were noticeably different ammonia removal capabilities.

Concentrations of N-TAN (Figure 5.11) during the day were particularly high (it exceeded 100 mg in certain moments in the case of the manual and automatic feeding) for the control diet which may be related to the maximum N-TAN concentration reported in Figure 5.10. There were no noticeable differences in mean N-TAN between feeding strategies for the experimental diets (FM and VM). The remaining nitrogenous substances ($N\text{-}NO_2^-$ and $N\text{-}NO_3^-$) were mainly high for the control diet as well for the auto-demand feeding strategies for the FM and the VM diet, consequence of the higher nitrification. In general, the rise and drop of the N-TAN and the $N\text{-}NO_2^-$ occurred for every combination of process parameters, although the ammonia peaks are more noticeable in the case of the manual and the automatic feeding strategy with limited feedings.

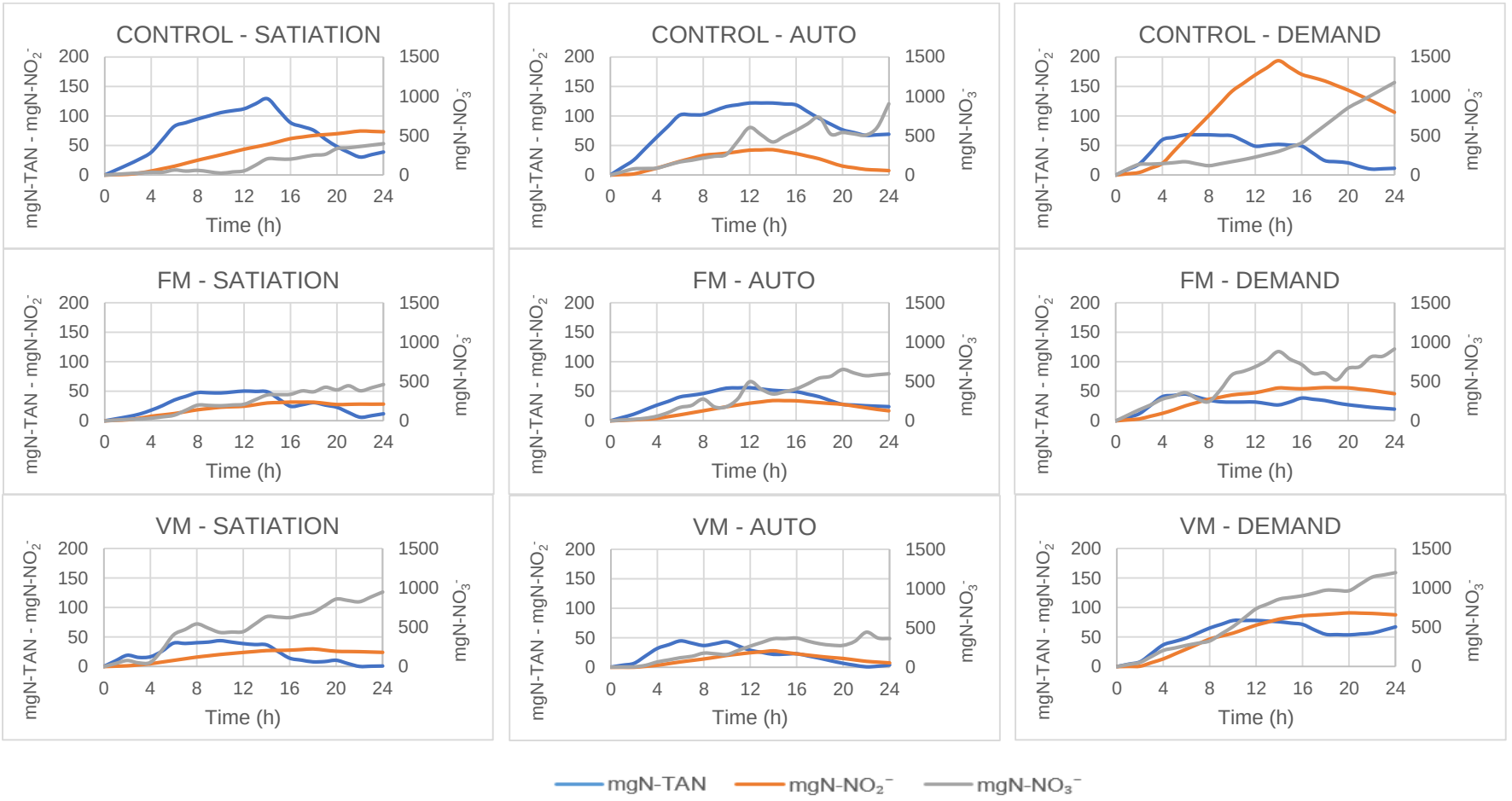

Figure 5.11. Concentration of nitrogenous compounds during the biofilter performance trials. Graph titles indicate the combination of variables whose influence on ammonia removal rates was investigated.

NO_2^- only showed an unexpected behavior for the combination of control diet and auto-demand feeding strategy, in which their peak was higher than for any other combination, suggesting a possible impairment of the nitrite oxidizing bacteria.

4. Discussion

This paper shows an essay of marine water fish production (*Sparus aurata* in this case) in a pilot-scale RAS with appropriately sized biofilters fed with experimental diets compared to a control diet, with focus on biofilter efficiency and harmful effects due to nitrogenous excretions. The size of the biofilters, as well as reactor specific parameters such as filter media and hydraulic loading were adopted from previous studies with optimal results (Godoy-Olmos et al., 2019, 2016).

Fishmeal replacement (VM diet) was apparently responsible for the significant differences in survival, specific growth rates as well as other nutritional aspects such as nitrogen retention. Diets without including only plant products as protein source have been proved detrimental to fish welfare (Estruch et al., 2018; Monge-Ortiz et al., 2018) in different scale RAS. On the contrary, both the control and the FM diet yielded satisfactory results when comparing SGR and FCR values with similar growth studies on RAS systems (Martínez-Llorens et al., 2012). Fish adapted reasonably well to all of the feeding strategies carried out on this study, with some brief periods of acclimation.

Nitrogenous waste characterization on the other hand was also similar to those found on the literature (Dalsgaard and Pedersen, 2011; Morales et al., 2018) for every nitrogen characterization measured on this study. Retained and excreted as TAN nitrogen was similar (around 30%), while the fraction of solid nitrogen was distinctly lower (3-12%). The main difference with those studies is that the diets considered in this study had very different digestibility and nutritional profile, which led to marked differences in solid nitrogen wastage, nitrogen retention and, to a lesser extent, N-TAN excretion between diets. There also were sources of nitrogen not accounted in the study (this is, nitrogen ingested was not equal to the sum to retained nitrogen + solid waste + dissolved waste). Among the unaccounted N sources, there are other nitrogenous substances excreted by fish, not determined in this study. Urea-N, for instance, may represent around 12% of the total nitrogenous excretion (Dalsgaard et al., 2015). Other unaccounted nitrogenous excretions have also been reported (10% according to Dalsgaard, Larsen and Pedersen (2015)), and is speculated to be composed by entire amino-acids (4-10%) or

mucoproteins (3-11%) which are excreted regularly (Kajimura et al., 2004; Wood, 2001). Differences in analytical methods explain deviations from total dissolved nitrogenous excretion and the sum of the individual components as well (Kajimura et al., 2004; Pedersen et al., 2012).

Nitrogen lixiviated from unconsumed feed is, unfortunately, a considerable source of unaccounted nitrogen for this study taken into account the characteristics of the pellets as well as the feeding strategies. Pellets were notably unstable in water (especially the experimental diets), therefore a certain degree of disaggregation of the diet is unavoidable. The relationship between stability and nutrient leeching has been discussed elsewhere (Fagbenro and Jauncey, 1995), as well as the influence of experimental ingredients in the stability of fish diets (Hoyos et al., 2017). This degree of disintegration is surely one of the reasons for the higher unaccounted nitrogen for the experimental diets (FM and VM) in comparison with the control diet, which was more compact. In addition, the specific characteristics of the auto-demand feeding strategy impedes unconsumed feed recollection, therefore it was decided not to collect and subtract unconsumed feed from the daily feed intake. Nevertheless, even if some feed is lost, it is assumed that the degree of feed lost is proportional to the amount of feed intake for a given feeding strategy and the proportion of feed lost is relative to the feeding strategy.

Fish weight did influence oxygen consumption as expected. Juvenile fish (which in the study coincided with the manual feeding strategy) consumed more oxygen per g of feed than on-growing fish, which is similar to other studies (Cerezo Valverde and García García, 2004; Steinarsson and Moksness, 1996). However, the oxygen consumption was significantly higher for the auto-demand feeding strategy regardless of taking mean weight as a co-variate, suggesting an effect of the use of auto-demand feeders by itself. Higher feed intake is responsible for increased oxygen consumption (Kajimura et al., 2004). Guinea and Fernandez (1997) found significant differences in oxygen consumption when feeding rate were increased from 1% of body weight to 2%, but did not find significant differences when the same ration (1% body weight) was distributed in one or two feedings. In our study there were no differences in oxygen consumption between the manual and automatic feeding strategy taking mean weight as a co-variable. Since there are few comparable experiments, it is quite difficult to determine which other effects derived from the feeding strategy could also be relevant, although the higher

metabolic effort invested in activating the mechanism to release the feed in the auto-demand feeders might be a possible explanation.

Diet also influenced oxygen consumption independent of feed intake, which may be attributed to general stress of the fish fed the plant meal based diet compared with the other two groups. Replacement of fish meal by plant protein sources lead to immunosuppression as well as an increment in oxidative stress, especially if replaced on its entirety (Sitjà-Bobadilla et al., 2005). The low welfare of the fish the VM group was probably responsible of the lower survival rate (Estruch *et al.*, 2018), which in itself also introduced an element of increased stress and therefore increased metabolic activity and oxygen demand. Finally, the inferior digestibility which is a factor of increased organic matter may also suppose an increment in oxygen consumption due to the increased BOD in biofilter influent (Fernandes et al., 2015).

The protein rich control diet led to the highest ammonia excretion without considering feed intake, while there were no differences between the remaining diets. Even if protein content of diets were similar, fishmeal based diets have been proved to produce higher ammonia excretion than plant based diets (Cheng et al., 2003; Obirikorang et al., 2015), therefore in our study most likely the combination of factors would have led to the highest excretion. However, when analyzing ammonia excretion by feed intake, ammonia excretion of the fish fed the VM diet was not significantly different than fish fed the control diet (Table 5.5). This is also pointed out in Figure 5.11, in which for the VM diet coupled with the auto-demand feeding strategy led to a higher ammonia concentrations than for the other feeding strategies due to the higher feed intake. It could be deduced, consequently, that the low total excretion (mgN-TAN kg biomass^{-1}) of the fish fed the VM diet was due to their lower feed intake rather than the quality of the diet.

The superior excretion rate of the fish fed the VM diet could be derived from the anti-nutritional factors present in them which affect protein digestibility (Francis et al., 2001; Kokou and Fountoulaki, 2018). The incomplete digestion of protein leads to the alteration of the bioavailability of amino acids, which coupled with the poor amino acidic balance of the proteins of plant origin ultimately leads to insufficient absorption. Berge *et al.* (2004) demonstrated that the absence of neutral amino acids impairs the uptake of essential ones like L-methionine. The inclusion of synthetic amino acids is suggested to improve the amino acidic balance of the plants without fishmeal (Monge-Ortiz et al., 2016), although it is suspected that their inclusion does not resolve the imbalances as

the free muscle amino acids measured after feeding do not compose the ideal amino acidic profile compared to fish fed fishmeal diets, at least for gilthead sea bream (Gómez-Requeni et al., 2004). On addition, fish fed plant-meal based diets are known to delay the digestion of those proteins, as evidenced by differences in post-prandial enzymatic activity, leading to different rhythms of absorption between synthetic amino acids and digestive amino acids (Santigosa et al., 2008).

Ultimately, if the amino acids absorbed do not meet the amino acidic balance required for protein retention, the excretion of unneeded nitrogen is an unavoidable consequence. These results may be also compared with the nitrogen balance (Figure 5.9), in which the percentage of the consumed nitrogen that was excreted was higher for the VM diet than for the FM diet. Fish fed the control diet, on the other hand, contain a surplus of protein that is being consistently used for energetic purposes, thus they show a high ammonia excretion regardless of feed intake, while for fish fed VM diet certain degree of feed intake is necessary for the excretion to take place.

Fish were fed to apparent satiation showed the highest ammonia excretion, independent of the consideration of the feed intake in the ratio. Restricted feeding strategies (manual feeding and automatic feeding are included in this category) prevent overfeeding and result in less feed wastage and better conversion indexes (Mihelakakis et al., 2002) but, especially for suboptimal diets, fish might increase their ammonia excretion as a result of not meeting their energetic needs (Kaushik, 1998). In contrast, the nutrient efficiency of non-restricted feeding strategies (auto-demand feeders) is usually higher, as fish are able to regulate their feed intake according to their appetite (Azzaydi et al., 1998). Pedrosa et al. (2019) reported decreased TAN excretion as a result, compared to manual feeding strategies. In our experiment, probably the increased total TAN excretion is caused by the increased feed intake that took place in that stage of the trial, but when feed intake is considered (Figure 5.8), the lowest ammonia excretion rate is a reflection of the lack of wastage of ingested feed.

Differences in ammonia excretion between the use of manual feeding and automatic feeding are probably the most difficult to explain. In our study, the feed intake was slightly higher when distributing the diet via manual feeding which might be the most influential factor on the differential excretion, although other considerations should be discussed. Restricted feeding periods have been proven to increase fish metabolism as a result of competition (Cutts et al., 1998), but the concrete differences between these two feeding

systems possibly would require further investigation on a physiological level to determine which one promotes the most competition. The time during which the feed is distributed (g/min) could be one of the most differentiating aspects between those feeding strategies, being much quicker for the automatic feeders. Lastly, it must be noted that even though is well known that ammonia excretion rate is higher in the early stages of growth, it did not seem to have an influence for our study (p-value>0.05).

Ammonia removal was, consequently, also highly dependent on diet and feeding strategy. In the case of restricted feeding strategies, there was a good correlation between the maximum ammonia/mean ammonia concentration and the ammonia removal, which is expected considering the range of ammonia concentration found on the rearing water (Henze, 2002). In contrast, in the case of the auto-demand feeders, ammonia removal rates were the highest compared to the remaining feeding strategies but ammonia concentration in water were not nearly as high, especially for the highest ammonia producing control diet. Not only were ammonia concentrations low, but they were more stable without any noticeable peaks, which has been recommended as well elsewhere (Eding et al., 2006) for the correct functioning of a recirculating aquaculture system. In sum, these factors seem to demonstrate the benefits of using auto-demand feeders for managing nitrogenous wastes., although probably the spreading of ammonia production (form few restricted feeding periods to a continuous feeding period) is one of the factors that prevents rapid accumulation of ammonia in water.

On the other hand, for practically every feeding strategy ammonia removal rates of the systems with fish fed the VM diet were comparably lower than for the other diets although the excretion rates are not always significantly lower. This highlights a negative influence of the plant meal based diet on the nitrifying capacity. Again, the lack of relatable literature impedes further exploration of the significance of this finding, but possibly the poor digestibility of the plant meal based favored the occurrence of organic matter in water and therefore shift the C/N ratio producing more favorable conditions for heterotrophic bacteria to outgrowth and outperform nitrifying bacteria. Davidson *et al.* (2013), in a similar study using experimental diets, found no significant differences in ammonia removal rates and the fishmeal diet only led a to a very slightly superior rate (69 vs 71) although the concentration of N-TAN was found to be generally higher in the RAS system for the fish fed a plant meal based diet. Besides, being the influent ammonia concentration higher for the plant diet systems, it would be expected to find a superior ammonia removal rate as their range of concentrations were optimal for ½-order

nitrification kinetics (0.58-0.77 mg/L), demonstrating both factors an inferior biofilter performance. In addition, the experimental diets, including the VM diet, were more granular and therefore they were more prone to partial dilution in water and not being consumed by fish. The solid removal systems of those tanks required more cleaning than the control tanks (especially in the auto-demand feeding phase) and this by itself on the long run makes the impairment of nitrifying bacteria more possible.

CONCLUSIONS

Growth of gilthead sea bream (*Sparus aurata*) is possible in pilot scale optimized recirculating aquaculture systems with biofilters sized to the predicted ammonia production of fish during its growth. Differences in growth were provoked by diet.

Oxygen consumption and ammonia excretion were influenced by diet and feeding strategy. Even though the fish growth stage majorly influenced oxygen consumption rates, there still was a significantly greater oxygen consumption rate when diet was distributed by auto-demand feeders, possibly derived from higher feed intake and increased energy use. Ammonia excretion rates were higher with the control diet and the manual feeding strategy. The higher protein content and the unfulfilling energetic and/or balanced protein requirements were most likely responsible for the increased ammonia excretion.

Ammonia removal rates were also dependent on diet and feeding strategy and only for the restricting feeding periods were proportional to ammonia loading as a consequence of increased excretion. For the auto-demand feeding strategy ammonia removal was the highest although the ammonia concentration maximum was not nearly as high as for the other feeding strategies with high removal rates, likely derived from the lack of peaks of ammonia excretion. Differences in diets' organic matter production might lead to differences in ammonia removal rates for fish with similar ammonia excretion, derived by outperformance of heterotrophic bacteria at the expense of nitrifiers.

GENERAL DISCUSSION

1. Influence of process parameters on the nitrification rate

The results of this thesis demonstrate the complexity of the interaction between factors affecting ammonia removal rates. Particularly every process parameter that was studied had a significant effect on the ammonia removal rate in some degree, summarized in Table 6.1.

Table 6.1. Summary of the effects of process parameters on the N-TAN removal rate

Parameter	Filter media	Temperature	Hydraulic loading (m^3/m^2 h)	OM	Response	Mean N-TAN removal rate (gN-TAN m^{-2} day^{-1}) ± SEM
Ammonia load	Bactoballs	Low (16-20ºC)	12	No	Not significant	0.165±0.017
			11	No	Significant	0.107±0.008
			8	No	Significant	0.103±0.007
			4	No	Significant	0.099±0.007
		High (20-27ºC)	12	No	Linear	0.297±0.060
			11	No	Significant	0.149±0.006
			8	No	Significant	0.150±0.006
			4	No	Significant	0.144±0.006
			9.81	Yes	Linear	0.075±0.005
	MECHpro	Low (16-20ºC)	12	No	Not significant	0.052±0.016
		High (20-27ºC)	12	No	Not significant	0.126±0.033
	Type A Biofill	Low (16-20ºC)	12	No	Linear	0.143±0.016
		High (20-27ºC)	12	No	Linear	0.366±0.056
Temperature	Bactoballs	N/A	12	No	Not significant	0.235±0.026
			11	No	Significant	0.143±0.008
			8	No	Not significant	0.137±0.008
			4	No	Not significant	0.104±0.006
	MECHpro	N/A	12	No	Not significant	0.078±0.014
	Type A Biofill	N/A	12	No	Exponential	0.223±0.028
Hydraulic loading	Bactoballs	Low (16-20ºC)	N/A	No	Not significant	0.105±0.007
		High (20-27ºC)	N/A	No	Significant	0.143±0.005
Diet	Bactoballs	High (22ºC)	9.81	Yes	Significant	0.075±0.005
Feeding strategy	Bactoballs	High (22ºC)	9.81	Yes	Significant	0.075±0.005

The effect of individual process parameters observed in this thesis is similar to those reported in literature. Low temperatures inhibited nitrification (Zhu and Chen, 2002), low hydraulic loadings hindered the ammonia removal capability (Kamstra et al., 1998) and the removal was greater the higher the ammonia concentration (Bovendeur et al., 1987; Greiner and Timmons, 1998), up until a certain threshold.

Notably, ammonia removal rates were very different depending in filter media. Not only in absolute value, but also in their dependence on temperature and ammonia loading. As new filter media are constantly being introduced on the market, our results highlight the irreproducibility of the nitrification rates obtained using specific filter media. Therefore, the general recommendation supported by the results of this thesis would be to evaluate the ammonia removal potential of every new filter media introduced as a carrier of a biofilter, including the analysis of the effects of the temperature and ammonia load (and possibly more) on the nitrification rates of biofilters containing such filter media. The results presented in Chapter 3 demonstrate than specific surface area alone is not enough to rate a filter media, as MECHpro® rings, in spite of its larger specific surface area compared to the other filter media, performed clearly worse than the other filter media tested, both in the ammonia removal rate values and in the TAN concentration in water. The discrepancy between a major specific surface area and greater nitrification rates have been also observed by Greiner and Timmons (1998), although they used two different types of biofilter (microbead vs trickling). Lekang and Kleppe (2000) tried several filter media of different characteristics and while a plastic media with fairly higher specific surface area (Kaldnes® rings) showed better performance, results were not corresponding by two of the filter media tested, whose performances were not directly related by their specific surface areas. Kishimoto et al. (2014) concluded in their experiments the roughness of the plastic media, a characteristic seemingly not previously considered in literature, as the sole source of a better nitrification capacity between two filter media.

When comparing new filter media, it seems reasonable to test their ability to remove different amounts of ammonia produced and establishing different water temperatures to make the results applicable for the largest number possible of aquaculture farms. In the filter media experiment (Chapter 3), hydraulic loading was equal for every recirculating unit and within the recommended range for trickling filters (12 m^3/m^2 h) (Timmons et al., 2009). However, for every filter media, two different biofilter volumes were tested in order to evaluate if a superior volume would be able to remove the

ammonia that a smaller biofilter could not. The calculation of ammonia removal rates, as performed throughout the thesis is not a good indicator of the convenience of biofilters with superior biofiltration areas, but maximum and/or mean N-TAN concentration in water are. Not surprisingly, there were no noticeable differences in maximum and mean N-TAN in water between the two biofilters containing MECHpro® rings despite the higher biofiltration area in one of them over the other. On the contrary, differences were found for the other filter media tested (Bactoballs® and Type A Biofill®), at least on the most favorable conditions. It is not very clear why MECHpro® rings offered the worst possible results. One possible reason, that was also reported by Lekang and Kleppe (2000), is that the apparent low void ratio of the MECHpro® rings produced the biofilter's clogging. Probably, if operated at a lower hydraulic loading or by back-flushing occasionally, the biofilters containing this filter media could have shown better performance.

The selection between Bactoballs® and Type A Biofill® as the best possible filter media requires more reasonable justification. The general mean ammonia removal rate was no significantly different between the two filter media and when analyzing the differences between ammonia removal rates for every combination of the remaining process parameters (TAN load and temperature), for some combinations one filter medium performed better while the exact opposite was true for other combinations of parameters. Being two very similar filter media in materials and shape (also in specific surface area and in void ratio), it is understandable that their potential for ammonia removal is very comparable. The higher ammonia removal rates achieved by Type A Biofill® under high temperatures could be explained by how the calculation was made, as the elimination of all of the ammonia added in every tank only leads to differential ammonia removal rates depending on the biofiltration area only and they were slightly different for every biofilter. Therefore, a higher TAN removal is caused by biofiltration area rather than intrinsic better capability for nitrification of the filter medium. In general, this is a particular case in which the superior specific surface area may have been determinant in achieving higher ammonia removal rates (for some combinations) but in practical terms both filter media could be considered equal and the selection should be based on additional traits (cost, weight, availability) rather than ammonia removal capabilities, which are also important in filter media (Lekang and Kleppe, 2000). When taking this into account, being able to construct biofilters with similar biofiltration areas with an inferior tank volume was a definitive factor for deciding on Bactoballs® over Type A Biofill® for successive experiments.

Chapter 4 further expands on the rating of Bactoballs® as filter media including a more extensive range of water temperatures, as well as different hydraulic loadings as process parameters influential to ammonia removal rates. Several of the conclusions provided in Chapter 3 are corroborated in this experiment, such as the inadequacy of low temperatures in achieving high ammonia removal. Another common trait is inability of removing the complete portion of TAN added at higher doses, while a small dose was practically removed in its entirety, provided the conditions were optimal. Regarding the different process parameter studied, the hydraulic loading, the deterioration of biofilter performance with decreasing hydraulic loadings was evident, although there were no significant differences in general between the two top hydraulic loadings, the highest of which was very similar (11 vs 12 m^3/m^2 h) to the one used in Chapter 3. The lack of wetting, pointed as one of the causes of bad performance (Nijhof and Bovendeur, 1990) was visually evident. Minimum and maximum hydraulic loadings for correct functioning of the biofilters have been reported elsewhere (Eding et al., 2006), which is comparable with the results provided in this thesis. Nevertheless, differences in ammonia removal rates between hydraulic loadings were non-significant at lower temperatures, while at the same time at high temperatures there were significant differences even between the top two hydraulic loadings, therefore it could be suggested that the maximum hydraulic loading that influences nitrification might be temperature dependent. In the consulted literature papers that do investigate the effects of hydraulic loading in ammonia removal (Greiner and Timmons, 1998; Peng et al., 2003) temperature is often only cited briefly and/or is kept constant throughout the experiment.

Although the actual ammonia removal rates reported for both experiments (using alike temperatures, ammonia loads and hydraulic loadings) are somewhat different, the difference in the vessel that held the biofilter is surely responsible for that, as well as the difference in analytical methods for ammonia determination. In Chapter 4, the experimentation with hydraulic loadings led to shape the biofilter in a specific manner as regulating water flow was not entirely precise. The materials were also different (the first vessels consisted in plastic trash cubes with holes in the bottom and the second vessels were plastic nets whose vertical “walls” were covered with a tarpaulin). It is possible that the hydraulic retention time was a little bit higher in the first experiment and/or there was some biofilm detachment, which are both important factors in nitrifiers’ activity (Nogueira et al., 2002).

For the biofilters analyzed in Chapters 3 and 4 some process parameters affected nitrification rate depending on other parameters; for example, in the case of the biofilters operating at low temperatures, the hydraulic loading was not significant to the performance of the biofilters as the temperature was responsible for low nitrification in general, but the opposite was true for the biofilters operated at high temperatures. These findings bring to light the lack of knowledge of the combined effects of several process parameters, which require specific research on the future. However, no opposing effects were discovered, and some combinations of parameters proved to be more beneficial than the sum of their positive influences. Even though characterization of relative importance of the parameters influential on the ½-order nitrification rate is occasionally discussed in literature (Eding et al., 2006; Lyssenko and Wheaton, 2006), the scientific assessment of the effects of combination of factors would be interesting as often it is impractical for commercial aquaculture to tailor the recirculating aquaculture systems to maximizing biofilter success and therefore compromises should be made.

2. Influence of organic matter in the effects of process parameters on nitrifying filter performance

In Chapter 3 and 4 the effect on particular process parameters were investigated and for this very reason the experimental setup aimed to keep the rest of the parameters that could affect nitrification on the same level between the six subsystems and thus ensure that the differences were caused by those parameters alone. This included organic matter (experiments were carried out by adding inorganic ammonia thereby limiting all interferences caused by heterotrophs including the consumption of dissolved oxygen), dissolved oxygen (was measured regularly and never was lower than 6 mg/L, thus ensuring no oxygen limitation), pH and alkalinity (pH variation was low during the 24-h trials and water was totally replaced before every trial), salinity and so on. The reasoning behind that was the selection of the best characteristics of a biofilter to use in a pilot scale recirculating aquaculture system in which fish were reared. However, it is known that the presence of organic matter may lead to the impairment of nitrification rates (Eding et al., 2006; Malone and Pfeiffer, 2006; Zhu and Chen, 2001), and in recirculating aquaculture systems it comes almost exclusively from fish nutritious wastes (Crab et al., 2007), therefore lower ammonia removal rates in the following experiment were to be expected, and thus were calculated as well, with the system in operation.

In that experiment (Chapter 5), ammonia removal rates were calculated using the differential ammonia productions caused by fish performance as the ammonia load

source rather than the addition of determined ammonia solutions in water. The performance was such that significantly different ammonia excretions were observed, and concentration of N-TAN was kept under 1.5 mgN-TAN at all times, a range of concentrations where the ½ order kinetic nitrification rate is likely to occur (Greiner and Timmons, 1998; Malone et al., 2006). For the ammonia removal rates calculated in Chapter 5, a general linear regression between the ammonia produced and the ammonia removed could be deduced (N-TAN removal = 0.00385104 + 0.00011981 * N-TAN excretion), with a relatively high R^2 (94.73%). The relationship between ammonia load and ammonia removal rate was thus similar to the previous experiments without including organic matter, which suggests that the effects of process parameters on nitrification possibly remain consistent independent on the C/N ratio, even if the actual nitrification rates are lower due to the influence of heterotrophic bacteria. For comparison, using mean values of N-TAN removal rates obtained at high temperatures, the highest hydraulic loading, using Bactoballs® as filter media (to remain consistent between the three experiments) and considering a TAN load of 3 g TAN/m^3 tank volume, without including organic matter a mean N-TAN removal rate of 0.147 and 0.10 gN-TAN/m^2 of biofiltration area and day was found in the first and second set of trials, respectively; whilst in the third experiment, made with live fish, a mean removal rate of 0.05 gN-TAN/m^2 of biofiltration area and day was found. Further experiments would be required to examine the influence of other process parameters (temperature, hydraulic loading) under different C/N ratios to corroborate if their influence is independent of the presence of heterotrophic bacteria, for more appropriate comparison between experiments.

3. Considerations about the relationship between fish nutrition and nitrifying filter performance

The ammonia excreted by the fish is very dependent on nutritional aspects such as the protein content of the diet, the feed intake and the feeding frequency (Ballestrazzi et al., 1994) and, on the other hand, uneaten and undigested feed are one of the factors which influences the most the amount of organic matter in water and therefore the C/N ratio. Both aspects serve as a demonstration of the importance of fish nutrition in improving and/or hindering the nitrification capability.

The importance of fish diet on biofilter efficiency have not been, to the best of our knowledge, sufficiently discussed. If live fish are used in experiments about biofiltration

performance or performance of a RAS in general, which in itself is rare, only the protein content of the diet (Terjesen et al., 2013) or feeding load (Pedersen et al., 2012) is usually taken into account. Even if is common that a diet with a notably higher protein content leads to higher ammonia excretion and higher N-TAN concentration in water (in our study the control diet led to such results), diets with similar protein content can also be responsible for very different ammonia excretion profiles, and more aspects need to be considered such as the amino acidic profile or the digestibility of nitrogen in general. On their own, there is a considerable amount of research articles analyzing ammonia excretion of diets containing different protein sources (Engin et al., 2013; Estruch et al., 2018; García-Romero et al., 2014; Obirikorang et al., 2015), but studies that integrate both analysis are scarce. A diet with low nutritional value, such as the plant-meal based diet used in Chapter 5 (VM) could be responsible of an increased ammonia excretion only in the case of a high feed intake, which also needs to be taken into account.

The organic matter load is also another important trait of biofilter performance. As stated earlier, studies have evaluated the effect of different C/N ratios on nitrification (Zhu and Chen, 2001), but it is not well documented which are the factors that could contribute to an increased C/N ratio rather than the use of different substrates for experimentation. However, diets with poor nutrient digestibility have been pointed out as a factor for the increase the organic loading in waste estimation (Davidson et al., 2016) and in bacterial community characterization experiments (Estruch et al., 2015). In Chapter 5 it is demonstrated that only the VM diet lead to inferior N-TAN removal rates although ammonia excretion was similar than for the fish fed the control diet and this might be attributed to the impairment of the nitrifying community via increased organic loading. In addition, oxygen consumption proved to be dependent on feed and it should also be considered when designing a biofilter to avoid an oxygen limitation that could be detrimental to both the fish and the biofilter, but specially to the biofilter because the aerators are usually placed in fish tanks and the excessive biological oxygen demand may produce oxygen shortages and reduced nitrification. This problem is maybe not that critical in trickling filters as they are self-aerated, but it is definitively worrisome for submerged filters. Therefore, another layer of analysis should be considered in the axis: diet characteristics -> ammonia excretion -> ammonia removal. In conclusion, those results would lead to the recommendation of, in the occasion of a variation in the fish feed used in an aquaculture plant (even if the protein content is similar), the re-estimation of the ammonia load and the ammonia removal capability of the biofilter for safety measures.

Feed distribution is another aspect rarely considered in biofilter performance trials. The exact moments and rations distributed throughout the day are not really considered for kinetic studies and performance models/calculations as the basis for ammonia removal/nitrification rates is often 24 hours and it is not really needed to determine empirically if the quantity of the nitrogenous waste produced is removed in its entirety. On the contrary, ammonia excretion determination on an hourly basis is laborious and difficult. Manuals and papers about biofilter design therefore only cite feed loading or feed loading based on protein content (Colt et al., 2006; Timmons et al., 2009). Biofilters, however, do not remove N-TAN in a constant rate as the removal is proportional to the influent concentration and therefore the reduction of the conversion to nitrate velocity is likely to shift as the ammonia is being depleted from the water. In the case of multiple feedings, ammonia is also expected to increase after each one according to the typical ammonia excretion profile (Echevarría et al., 1993). It is not very clear if the continuous decrease and increase of the influent concentration is beneficial or detrimental to the nitrification velocity, but the results of the present thesis appear to point to the first case.

The use of auto-demand feeders led to a continuous N-TAN concentration and also produced significantly higher ammonia removal rates than those achieved by manual and automatic feeding. Additionally, lower maximum N-TAN concentrations that for any other combination of process parameters tested in any other experiment prior, even considering the impairment of nitrification caused by the presence of heterotrophic bacteria, at comparable ammonia loads. In any case from the previous experiments, focused in determining the best possible configuration of the biofilter, the ammonia entered the fish tank in brief periods and ammonia peaks were observed, indicating an inability of fast removal of ammonia. Thus, the use of auto-demand feeders is recommended based on the results on this thesis as a manner of keeping an active biofilter removing a steady concentration of TAN without increasing biofilter and/or tank volume. The use of auto-feeders and their impact on fish performance from a productive point of view has been already discussed (Attia et al., 2012; Gómez-Peñaranda, 2005; Madrid et al., 2009), but to our knowledge there are no research articles related to nitrogenous excretion management including them as the feeding strategy used to feed the fish.

4. N-TAN concentration patterns: discussion about the performance measurements

N-TAN concentrations, as well as nitrite and nitrate in Chapter 4 and 5, were determined every two hours during 24 hour periods in which fish were fed as usual, or in the case of the "best configuration of biofilter" experiments, ammonia was loaded into the tank in the form of ammonium chloride. The objective was to monitor the changes in N-TAN concentrations throughout the day with the biofilter in operation, and to evaluate the maximum and minimum N-TAN concentrations achieved, as well as, in the first experiment, to determine if a superior biofilter volume could reduce the maximum ammonia concentration.

The direct relationship between ammonia influent concentration is well documented in RAS textbooks (Timmons et al., 2009) based on experiments performed without live fish (Greiner and Timmons, 1998) or calculating the filter efficiency with mass balances measuring the ammonia concentration in the inlet and the outlet of the biofilter (Michaud et al., 2006; Sandu et al., 2002; Terjesen et al., 2013). That could encourage aquaculturists to increase fish density and/or feed loading to take advantage of the full capabilities of the biofilter and reduce operational costs. However, due to experiments investigating the post-prandial ammonia excretion (Dosdat et al., 1996; Engin et al., 2013), it is known that a sudden rise of ammonia excretion is supposed to take place a couple of hours after feeding. The higher the protein content (and consequently the ammonia load), the higher the maximum concentration of these known "ammonia peaks" usually is. Therefore, it should be interesting to evaluate the velocity of ammonia removal of nitrifying filters, in their different configurations, in order to determine potential risks of fish welfare during the immediate hours post-feeding. In Chapter 3 and 4 an equivalence between the ammonia load and the maximum N-TAN concentration is presented, which questions the convenience to increase ammonia load to achieve higher nitrification rates, since the performance of the biofilter would not be optimal if the biofilter does not resolve fish welfare issues related with high ammonia concentration in water. In Chapter 3 peaks of N-TAN concentration are observed even if the biofilter is able to completely remove the ammonia added.

On the other hand, the determination of minimum N-TAN concentrations is also important to ensure the total elimination of the ammonia load of the day. If a higher N-TAN removal rate, consequence of higher ammonia influent concentration, is not enough to completely remove all the ammonia added during the day, over time a system outbreak and failure may occur. The improvement of ammonia removal with increasing concentration may

help prevent the over-accumulation of ammonia over time, but only in the case that the biofilters are not operating at their maximum capacity. In fact, in Chapter 3 it is also observed that in near biofilter saturation (this is, at high ammonia loads) the more voluminous biofilters did not led to significantly lower ammonia concentrations. Paradoxically, only at lower concentrations the biofilters whose biofiltration area was the highest reduced ammonia more quickly leading to drops in maximum N-TAN concentration.

In Chapter 5 an interesting trend was found when using auto-demand feeders, at least for Gilthead sea bream (*Sparus aurata*). The spreading of the feeding was most likely responsible for the continuous load of TAN throughout the day and no clear ammonia peaks were determined as a consequence. Besides, ammonia removal rates were significantly higher than those observed when feeding the fish manually or by using automatic feeders. This feeding strategy marks the exception in the correspondence between ammonia load, maximum N-TAN concentration and ammonia removal rate, which seems like an excellent starting point to maximize both biofilter efficiency and fish welfare, although it may not be applicable for other species with different feeding rhythms (Gómez-Peñaranda, 2005).

5. Possible future contributions of maximization of trickling filter performance

This thesis points out the complexity of the interaction of process parameters with every different filter media, therefore performance estimations should be carried out for every new filter media introduced. This thesis develops an easy and fast calculation method of the variables transcendental to the correct functioning of a nitrifying filter: ammonia removal rate (if possible by calculation of individual hourly ammonia removal or at least by safeguarding the total elimination of the ammonia load) and maximum N-TAN concentration and minimum concentration. The report of these factors might be more important that specific surface rate or other similar traits of filter media. In any case, those data should be documented for each ammonia load and temperature of interest. There is seemingly no compromise in designing future biofilters operating at the hydraulic loading that led to the best results in our thesis other than maybe the pumping requirements, therefore every experiment should be carried out at the most optimal hydraulic loading.

This thesis also brings to light the importance of nutritional aspects on the ammonia excretion and ammonia removal, and the continuous research about the replacement of fish meal makes this topic extremely important for the proximate future. The relationship between diet and ammonia removal rate has been found empirically in this thesis and, even though reports of microbiota changes based on the inclusion of plant-protein sources have been published (Estruch et al., 2015), the characterization of the bacterial communities inside the biofilter would definitively bring to light the influence of those replacements in the biofilter microbiota. Similar characterizations of biofilter microbiota depending on feeding strategy (or more accurately on feeding distribution) could also be interesting in order to analyze if there are advantageous effects on nitrifiers activity.

Nevertheless, the feeding strategy that yielded the best result in this thesis was the use of auto-feeders, and the spreading of the ammonia excretion was probably a consequence of the feeding habit of the species reared (Gómez-Peñaranda, 2005), the gilthead sea bream (*Sparus aurata*). There is no evidence that other species would reproduce the feeding habits of the fish reared in our study. However, the incorporation of analysis of ammonia excretion and determination of ammonia removal rate in growth experiments could be a simple instrument to provide more data on the ammonia excretion patterns of more species with different diets and, if possible, slight variations of the feeding strategy used in Chapter 5 could be carried out, such as using a fixed amount of feed, for instance, in the auto-demand feeders.

CONCLUSIONS

- The influence of process parameters on the ammonia removal rate as a measure of biofilter performance is observed with the objective of selecting the best parameters for the achievement of the highest ammonia removal rate

- Quantification of the influence of filter media was not possible but, at the current hydraulic loading rates, the mean lowest ammonia removal rate was observed by biofilters containing MECHpro® rings as filter media. The remaining filter media proved to be more efficient depending on particular conditions. Bactoballs® produced more consistent N-TAN removal rates across every ammonia load and temperature, but mean N-TAN removal rate at high temperatures were superior for filter containing Type A Biofill®.

- Low temperatures hindered nitrifications and were responsible for low ammonia removal rates across the first two experiments. Variations of ammonia load or hydraulic loading did not result in differential ammonia removal rates when the water temperature was low in the first two experiments.

- The effect of ammonia loading was significant in every one of the experiments. The higher the ammonia load, the higher the ammonia removal rate when the former was 6 g TAN/m^3 or lower.

- Biofilter containing Bactoballs® (for ability to rapidly reduce the ammonia load, price and specific surface area), operating at a hydraulic loading of 12 m^3/m^2 h and operating at high temperatures was selected as the most optimal configuration for a trickling filter, and therefore it was implemented in pilot recirculating aquaculture systems rearing gilthead sea bream (*Sparus aurata*)

- Ammonia excretion depended on protein content and nutritional value of the diet. In general, a higher ammonia excretion was responsible for higher ammonia removal rates, but those were hindered by the feeding with the VM diet and they were enhanced with the use of auto-demand feeders with "*ad libitum*" feeding. The organic matter content due to the poor digestibility of the VM diet might favor the heterotrophic bacteria growth and the impairment of the AOB and NOB. The improvement of the nitrification rates using auto-demand feeders is attributed to the spreading of the feeding which keeps a constant TAN concentration.

- Dissolved oxygen consumption was also increased due to the feeding with the plant meal based diet, attributed to the general stress from nutritional imbalance of the diet. The application of those diets in recirculating aquaculture systems may require additional aeration to ensure an appropriate oxygen flow to the biofilter.

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