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Microalgae-bacterial biomass outperforms PN-anammox biomass for oxygen saving in continuous-flow granular reactors facing extremely low-strength freshwater aquaculture streams

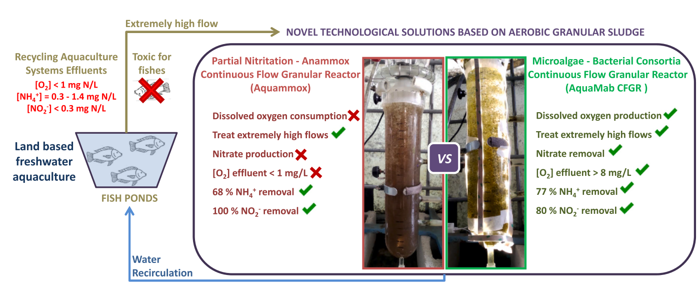
Sergio Santorio [a,*], Angeles Val del Rio [a], Catarina L. Amorim [b], Ana T. Couto [b], Luz Arregui [c], Paula M.L. Castro [b], Anuska Mosquera-Corral [a]

[a] CRETUS Institute. Department of Chemical Engineering, Universidade de Santiago de Compostela, E-15705, Santiago de Compostela, Spain
[b] Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005, Porto, Portugal
[c] Grupo Tres Mares, S.L. Lires S/n, E-15270 Cee, A Coruña, Spain

HIGHLIGHTS

- Microalgae-Bacteria granules greatly succeeded over PN-AMX granules.
- PN-AMX granules facing recirculating aquaculture effluents lost their anammox activity.
- Aerobic granules bioaugmentation with microalgae favoured the treatment process.
- Microalgae attachment to granules allowed their retention in the continuous flow system.
- Treated effluents meet the requirements for water recirculation in the aquaculture farm.

GRAPHICAL ABSTRACT



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ABSTRACT

The dissolved oxygen (DO) concentration in water streams is one of the most important and critical quality parameters in aquaculture farms. The main objective of this study was to evaluate the potential of two Continuous Flow Granular Reactors, one based on Partial Nitrification-Anammox biomass (Aquammox CFGR) and the other on Microalgae-Bacteria biomass (AquaMab CFGR), for improving dissolved oxygen availability in the recirculation aquaculture systems (RAS). Both reactors treated the extremely low-strength effluents from a freshwater trout farm (1.39 mg NH_4^+-N/L and 7.7 mg TOC/L). The Aquammox CFGR, removed up to 68% and 100% of ammonium and nitrite, respectively, but the DO concentration in the effluent was below 1 mg O_2/L while the anammox activity was not maintained. In the AquaMab CFGR, bioaugmentation of aerobic granules with microalgae was attained, producing an effluent with DO concentrations up to 9 mg O_2/L and removed up to 77% and 80% of ammonium and nitrite, respectively, which is expected to reduce the aeration costs in fish farms.

* Corresponding author.
E-mail address: sergio.santorio@usc.es (S. Santorio).

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1. Introduction

Aquaculture activities have been rising exponentially in the last decades. From 1990 to 2012, aquaculture production increased by 40% (Krause et al., 2015). Nowadays, the aquaculture industry produces approximately 46.8% of the total fish consumed in the world, amounting to 179 million tonnes in 2018 (FAO, 2020). In particular, the land-based freshwater aquaculture sector uses large volumes of water which, in warm and dry regions, is more likely to become a scarce resource. The lack of this resource would impair the farm productivity. Thus, recirculating aquaculture systems (RAS) are widely applied in land-based freshwater aquaculture farms, in order to reduce water needs and water dependence, boosting aquaculture production while alleviating environmental impacts such as eutrophication (Martins et al., 2010). Different studies point out that the use of RAS in intensive rainbow trout farms could enhance fish production (Pulkkinen et al., 2019, 2018; Suhr and Pedersen, 2010).

The success of RAS depends on the quality of the water that is produced to be further used to refill the fish tanks. The dissolved oxygen (DO) concentration of the water in fish tanks, is critical both to health and growth performances. Thus, in intensive aquaculture farms the water DO level should be continuously monitored. When the RAS operate in closed circuit, the DO concentration throughout the water stream decreases whereas pollutant concentrations increase (Crab et al., 2007). The DO concentration should be high enough to guarantee adequate fish growing conditions. For the production of salmonids, like rainbow trout, the DO should be over 6–8 mg O_2/L (Ebeling and Timmons, 2012). Moreover, low DO concentrations reduce fish respiration efficiency, especially if subjected to high carbon dioxide (CO_2) concentrations. As water oxygenation is costly, the maintenance of adequate DO levels in the water is important for both technical and economic reasons. Apart from DO, the presence of pollutants like nitrogen compounds needs to be carefully monitored and controlled. Among them, ammonium is the main form of nitrogen accumulating in the waterflow in RAS, which is easily oxidized to nitrite in aerated fish tanks. Free ammonia (FA) and free nitrous acid (FNA) accumulation, which depends on ammonium and nitrite concentrations, cause fish mortality by hypoxia because they diminish haemoglobin's capacity to transport oxygen (Russo et al., 1981). To reduce ammonium and nitrite concentrations (and consequently FA and FNA, respectively), the use of nitrifying biofilters in RAS is widely applied (Martins et al., 2010). As nitrification consumes oxygen, the water produced by those biofilters should be aerated to ensure enough DO concentration in the returning stream, to maintain fish health. However, oxygenation processes largely increase farm operational costs, representing about 13% of the total cost of a fish farm facility (Ebeling and Timmons, 2012).

On the other hand, to treat the large water flows used in aquaculture with a relatively low environmental footprint, compact systems need to be developed to minimize the surface area needed for their implantation. For this reason, novel technologies based on aerobic granular sludge (AGS) are being studied to remove nitrogen compounds as an alternative to traditional biofilters in RAS (Santorio et al., 2022, 2021). Santorio et al. (2022) demonstrated that pilot-scale continuous flow granular reactors (CFGR) with AGS can remove up to 81% of ammonium and 100% of nitrite present in the incoming water stream operating at an hydraulic retention time (HRT) of 30 min. However, as the effluent DO concentration was below 1 mg O_2/L, it implied the need for an oxygenation/re-aeration step similar to traditional RAS biofilters. Therefore, alternative technologies based on granular sludge need to be explored to simultaneously produce water streams with adequate levels of dissolved oxygen and nitrogen, which can then lead to more acceptable economic practices in land-based aquaculture.

The combination of partial nitritation and anammox (PN-AMX) processes lead to important reductions of DO requirements compared to conventional nitrification–denitrification processes (Jetten et al., 2005). In PN-AMX systems, only 50% of ammonium oxidation to nitrite is needed to convert the remaining nitrite and ammonium anaerobically to nitrogen gas. Thus, the oxygen consumed by PN-AMX processes is three times lower than that required for the conventional nitrification-denitrification processes (Van Hulle et al., 2010). The existence of anammox bacteria in moving bed biofilters treating freshwater recirculating aquaculture systems has been proved by van Kessel et al. (2010). Thus, the application of PN-AMX processes with granular sludge could be an interesting alternative to reduce the oxygen consumption in freshwater RAS. In addition, several studies demonstrated the potential of PN-AMX processes to treat low strength wastewater at low temperatures (Gilbert et al., 2014; Hendrickx et al., 2012; Isanta et al., 2015). However, to the best of the authors' knowledge, no research study has tackled the treatment of extremely low loaded water streams such as that of freshwater aquaculture in a PN-AMX system.

Another alternative to reduce the costs of water oxygenation is the use of systems based on Microalgae-Bacteria biomass (Ahmad et al., 2019; Huang et al., 2015; Quijano et al., 2017). This consortium creates symbiotic relationships that benefit both microbial populations. While nitrifying and heterotrophic bacteria employ the oxygen generated by microalgae through photosynthesis, the CO_2 generated by bacterial respiration is, in its turn, used in microalgae metabolism (Zhang et al., 2021). Moreover, it is expected that the DO produced by microalgae photosynthesis is enough for the metabolic processes of bacterial populations in non-aerated reactors (Foladori et al., 2018; Petrini et al., 2018; Yang et al., 2018). Thus, in comparison with conventional AGS applied to wastewater treatment, which comprise mainly bacterial populations, the use of the Microalgae-Bacteria consortium allows the reduction of oxygenation costs while simultaneously minimizing CO_2 emissions (Zhang et al., 2021).

When the treatment of aquaculture effluents is established only on microalgae based processes, it is necessary to operate the system at long HRT (1 day or longer) to allow for enough biomass in the system to achieve good pollutant removal performances (Liu et al., 2019). Thus, the use of microalgae in the form of granules could be an interesting alternative to retain the microalgae in the system allowing to perform at shorter HRT. Fan et al. (2021) applied Microalgae-Bacteria granular sludge to treat aquaculture water achieving an ammonium removal efficiency of 85% in a sequencing batch reactor (SBR). Ahmad et al. (2019) studied the performance of a Microalgae-Bacteria granular continuous reactor without aeration supply, but a poor nutrient removal performance was achieved after stopping aeration, leading to the need of applying intermittent aeration to recover the system's efficiency. Moreover, Ji et al. (2022) demonstrated the feasibility of treating synthetic aquaculture streams (11.4 mg NH_4^+-N/L, and 9.9 mg NO_2^--N/L) using a non-aerated continuous-flow tubular reactor based on Microalgae-Bacteria granular sludge achieving removal percentages up to 92% and 98% of ammonium and nitrite, respectively. However, the reactor was operated at an HRT of 6 h, unsuitable for the treatment of extremely high flows of some freshwater aquaculture farms.

The aim of the present research was to study the performance of two reactors based on granular sludge composed by PN-AMX biomass and Microalgae-Bacteria biomass. In the case of the PN-AMX reactor, the main challenge is to maintain these processes facing extremely low concentrated aquaculture streams, an issue never explored before to the best of the author's knowledge. The main challenge in the Microalgae-Bacteria reactor was to retain the microalgae in the system when operating at short HRT, to face the extremely large flows produced in the aquaculture farms. In fact, the retention of microalgae facing HRT lower than 40 min has not been yet achieved.

We hypothesise that both proposed treatment alternatives are low environmental footprint systems that require low oxygen consumption to produce water streams with chemical quality, and thus are promising and feasible systems to be applied in RAS in a rainbow trout farm. Therefore, in an *in situ* approach, the systems were operated in continuous mode and at short HRT (<40 min) for the treatment of extremely low-strength effluents produced in a trout farm (<1.4 mg NH_4^+-N/L). As nitrogen and DO concentrations are critical water quality parameters for water recirculation in aquaculture farms, the present research mainly focused on both requirements to evaluate the suitability of both proposed systems.

2. Materials and methods

2.1. Experimental setup and operational conditions

Two laboratory-scale reactors were operated *in situ* in a rainbow trout farm (salmonids) for the treatment of extremely low-strength freshwater aquaculture recirculating wastewater. The reactors were run consecutively during the summer period - first the Aquammox CFGR for 67 days and afterwards the AquaMab CFGR for 33 days. The AquaMab reactor was started up later as the bacterial granular biomass used as inoculum came from a pilot plant which was in operation simultaneously, and needed 47 days to form granules. The length of operation of the AquaMab CFGR was shorter than that of the Aquammox CFGR due to the premature beginning of the rainy period that caused influent concentration dilution and decreased the water temperature to below 10 °C, and impeding the conditions required for the purpose of the AquaMab operation.

Both reactors consisted of a 2.3 L cylinder with a 3-phase (gas-liquid-solid) separator in the upper zone. Mixing in both reactors was achieved by mechanical stirring (40–100 rpm) and no aeration was provided. The trout farm water stream was continuously pumped into the reactor from its bottom. The treated effluent was discharged from the reactor upper zone by liquid overflow. Temperature, pH and DO concentration inside the reactors were measured but not controlled, mainly depending on the trout farm water streams produced (Table 1). For the AquaMab CFGR operation, a white LED light strip (300 lumens) was placed around the reactor walls to promote microalgae growth. The LED light was connected continuously to provide light over all the experiment (without dark phase) and guarantee the photosynthesis.

Since treating flows as high as possible is mandatory to operate reactors in freshwater aquaculture farms, short HRT were imposed to both reactors. The Aquammox and AquaMab HRTs ranged from 14.4 to 38.3 min and 30.7–86.8 min, respectively. With this stress conditions, only the biomass able to aggregate in flocs and/or granules could remain inside the reactor. The DO concentrations in the incoming water streams (3.9–10.8 mg O_2/L) should be enough to promote the nitrifying activity, without the necessity of external aeration.

2.2. Seeding sludge and microalgae bioaugmentation

The Aquammox CFGR was inoculated with PN-AMX granular biomass from a full-scale ELAN® reactor treating the effluent from an anaerobic sludge digester of a municipal wastewater treatment plant located in Guillarei (Tui, Spain) (Morales et al., 2015). The initial seeding sludge concentration inside the reactor was of 3.6 g VSS/L and it presented a Sludge Volume Index (SVI) of 53 mL/g TSS. The specific anammox activity (SAA) of the seeding sludge was of 352 ± 48 mg N_2–N/(g VSS·d).

The AquaMab CFGR was inoculated with AGS from a pilot-scale CFGR treating the water from the water stream in an aquaculture fish farm located in Lires (Cee, Spain), as in Santorio et al. (2022). The initial seeding sludge concentration inside the reactor was 0.6 g VSS/L, presenting a SVI of 105 mL/g TSS. The specific denitrifying activity (SDA) of the seeding sludge was 51 ± 4 mg N_2–N/(g VSS·d). Initially, the AquaMab CFGR was seeded only with AGS from the pilot-scale reactor and after 8 days, the suspended microalgae consortium composed by strains isolated from the facility (Couto et al., 2021) was added. The microalgae consortium was composed by different genera of the Chlorophyta phylum. The bioaugmentation was performed by inoculating 1.4 L of a suspended microalgae consortium with a concentration of 0.25 g VSS/L. Due to its poor settling properties, the microalgae culture was added to the AquaMab CFGR in one pulse and the influent feeding was stopped for 12 h to promote its retention inside the reactor and to avoid biomass wash out. During this period the mechanical stirrer continued working, maintaining the reactor mixing to favour the contact between microalgae and granules. After 12 h the reactor was fed again continuously, and the HRT was progressively decreased till reaching the value of 30.7 min (Vup = 0.7 m/h) after 16 days of the microalgae addition.

Table 1
Operational conditions of the Aquammox and AquaMab CFGRs.

	Aquammox CFGR	AquaMab CFGR
Operation length (days)	67	33
Type of biomass	PN-AMX	Microalgae-Bacteria
[a]T (°C)	17.3–25.5	11.4–22.3
[a]DO (mg O_2/L)	3.9–8.4	5.4–10.8
[a]pH	5.3–6.2	6.0–6.4
[a]TOC (mg/L)	4.16–7.71	3.34–6.07
[a]NH_4^+-N (mg N/L)	0.35–1.39	0.34–0.71
Feeding flow (L/h)	3.6–9.6	2.7–4.6
HRT (min)	14.4–38.3	30.7–86.8
Up-flow velocity (m/h)	0.6–1.5	0.4–0.7

DO: Dissolved Oxygen; HRT: Hydraulic Retention Time; T: Temperature; TOC: Total Organic Carbon.
[a] Values of the influent stream.

2.3. Metabolic batch activity tests

The maximum SDA was determined according to the manometric method described by Buys et al. (2000), in vials of 35 mL, incubated at 20 °C. The initial concentration of substrates was of 50 mg NO_3^--N/L and 225 mg COD/L (acetate) inside the vials, resulting in a C/N ratio of 4.5 g/g. All these batch activity tests were conducted in triplicate.

The maximum SAA was determined according to the manometric method described by Dapena-Mora et al. (2007), in vials of 35 mL, incubated at 20 °C. The concentration of ammonium and nitrite were of 70 mg N/L each. The overpressure inside the vials was measured with a differential pressure transducer, 0–5 psi range and linearity 0.5% of full-scale, Centerpoint Electronics. The biogas composition was determined with a gas chromatograph Hewlett Packard 5890 series II. All these batch activity tests were conducted at 20 °C, in triplicate.

The respirometric assays were performed only for the AquaMab CFGR biomass to determine the maximum specific ammonium oxidation activity and the maximum specific microalgae oxygenation activity. A 250 mL Pyrex® vessel was used to measure both activities following the method developed by Vargas et al. (2016) providing light with a 300 lumens LED device. For this purpose, the biomass was first submitted to aeration to reach DO saturation conditions, then the aeration was stopped and the metabolic activities were measured in the following order: (1) endogenous activity in dark conditions, which promotes bacterial oxygen consumption for metabolism; (2) maximum oxygenation activity of the microalgae present in the biomass, measured in the presence of light; (3) AOB activity determined in dark conditions, with addition of ammonium source; (4) oxygenation capacity of the Microalgae-Bacteria consortium when AOB activity is present, with addition of ammonium source and presence of light. A portable oximeter with a LDO Hach Lange probe was used for the data acquisition. The dark conditions were imposed by covering the vessel with aluminium film and turning off the LED strip to ensure the absence of light.

2.4. Analytical methods

Influent and effluent streams of both reactors were sampled 2–3 days a week to follow the reactors' performance. Liquid samples were filtered using cellulose-ester filters (0.45 µm pore size) to remove suspended solids. A spectrophotometric method was applied to determine the ammonium concentration (Bower and Holm-Hansen, 1980). Nitrite and nitrate concentrations were determined according to the Standard Methods (APHA/AWWA/WEF, 2012). Total organic carbon (TOC) and inorganic carbon (IC) concentrations were determined by a Shimadzu analyser (TOC-L, automatic sample injector Shimadzu ASI-L). DO concentration was measured using a luminescent DO probe (LDO, Hach Lange). The pH was determined with an electrode connected to a Hach Sension^{+} meter. Average diameter and size distribution of the AquaMab and Aquammox granules were determined using a stereomicroscope (Stemi, 2000-C, Zeiss), incorporating a digital camera (Coolsnap, Roper Scientific Photometrics). The obtained images were processed using Image ProPlus® software.

2.5. Mass balance

The nitrogen mass balance was described in equation (1) (Eq. (1)) where the nitrogen (as ammonium and nitrite) consumed match with the nitrate produced.

$$N - NH_4^+ \text{consumed} + \quad N - NO_2^- \text{consumed} = \quad N - NO_3^- \text{produced} \qquad \text{Eq. 1}$$

If both sides of the equation match, the occurence of nitrification was confirmed, otherwise indicates the occurrence of denitrification or nitrate assimilation by microalgae.

3. Results

3.1. Nitrogen transformations

In the Aquammox CFGR, during the first 42 days of operation, the ammonium and nitrite influent concentrations ranged between 0.71 and 1.39 mg NH_4^+-N/L and 0.04–0.87 mg NO_2^--N/L, respectively (Fig. 1a).

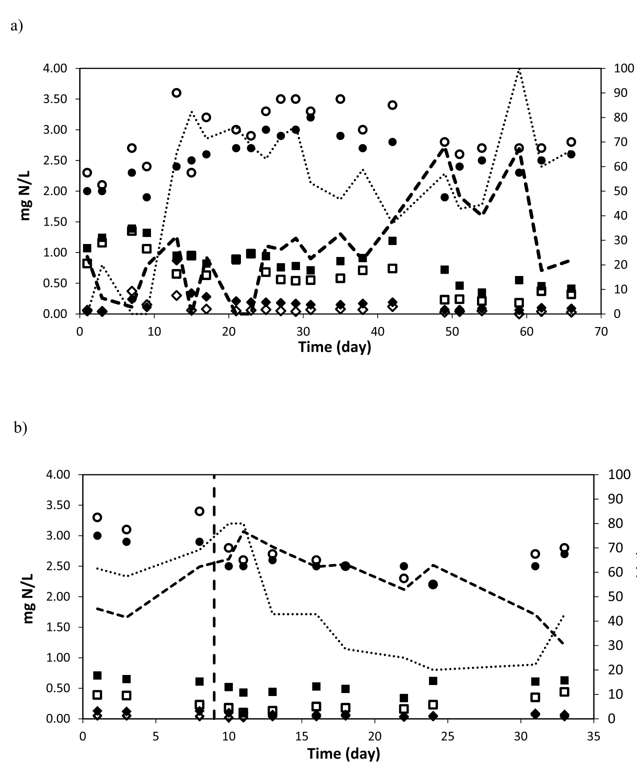

Fig. 1. Ammonium influent (■) and effluent (□) concentrations, nitrite influent (◆) and effluent (◇) concentrations, nitrate influent (●) and effluent (○) concentrations, ammonium (—) and nitrite (⋯) removal percentages in the Aquammox CFGR (a) and in the AquaMab CFGR (b). The dashed line in figure b splits the operational period before and after microalgae bioaugmentation.

During the first 23 operational days, ammonium removal was unstable, between 0 and 32%. Afterwards, ammonium removal became more stable, ranging from 22 to 38%. From day 42 onwards, with the decrease of the ammonium influent concentration to values below 0.4 mg NH_4^+-N/L, the removal percentages varied within the range of 18–68%.

Nitrite removal performance followed a similar behaviour and the achieved removal percentages were higher than those of the ammonium removal. During the first operational days, the nitrite removal was between 0 and 82%. From day 22 until the end of operation, nitrite removal was in average approximately 70%, reaching values up to 100%.

Nitrate was produced in the Aquammox CFGR, as the concentration of this nitrogen form in the effluent was higher than in the feeding stream as it could be seen on operational days 13, 42, and 49 (Fig. 1a). The nitrate production was directly correlated with ammonium and nitrite disappearance (Gujer, 2010; Yang et al., 2014). Mass balances indicated that the sum of the consumed ammonium and nitrite corresponded to the nitrate concentration increase in the effluent, indicating that complete nitrification was the process taking place instead of the partial nitritation and anammox processes.

In the AquaMab CFGR, before the microalgae addition (first 8 days of operation), the ammonium and nitrite removal percentages were around 45–60% and 60–75%, respectively (Fig. 1b). During this period, nitrate concentrations in the effluent were higher than those in the influent. The mass balances revealed that the increase of the nitrate content in the effluent corresponded to the decrease of the ammonium and nitrite contents in the influent. After the microalgae addition, the ammonium and nitrite removal percentages slightly increased, ranging from 53 to 77% and 20–80%, respectively. However, the nitrate-nitrogen in the effluent was below the value of the sum of ammonium- and nitrite-nitrogen amounts in the influent. From day 18 to day 24, the nitrate-nitrogen concentration in the effluent was below the one of the influent, corresponding to a 15% of the total nitrogen (TN) removal.

3.2. Organic and inorganic carbon removal and pH profile

In the Aquammox CFGR, the TOC concentration in the influent ranged between 4.1 and 7.7 mg C/L throughout all the operational period. Only in the last days of operation, from day 40 onwards, TOC removal occurred (Fig. 2a), displaying TOC effluent concentrations higher than those in the influent. TOC effluent concentration increase was up to 2.5 mg C/L. Regarding inorganic carbon (IC), the influent concentration decreased progressively from 2.71 to 0 mg C/L from the start-up to day 29, remaining like this until day 42. Afterwards, the IC influent concentration increased in the range 0.57–0.91 mg C/L, until the end of the operation. In the Aquammox CFGR, the IC was consumed at the percentages of 7–34%, in the first 29 days. From day 42 onwards, this removal increased to 100% (Fig. 2a).

In the AquaMab CFGR, the TOC concentration ranged between 3.1 and 6.3 mg C/L during all the operation, and the TOC removal stood between 0 and 18% (Fig. 2b). The IC influent concentration was around 0.9 mg C/L most of the time, and its consumption was approximately 100% (Fig. 2b).

The pH in the influent ranged between 5.3 - 6.2 and 6.0–6.4 in the Aquammox and AquaMab reactors, respectively (Fig. 2c and d), which is similar to the average pH of the natural freshwater stream in the region. In the Aquammox reactor, the pH in the effluent was approximately 0.1–0.7 units lower than that in the influent during most of the operation (Fig. 2c). Only in the first 7 days of operation the opposite was observed. In the AquaMab reactor, two different behaviours were observed. During the first 16 days, the pH was lower in the effluent and this pH decrease was higher before microalgae addition. From day 18 onwards, the pH values in the influent and effluent were similar, and eventually it was higher in the effluent (day 24) (Fig. 2d).

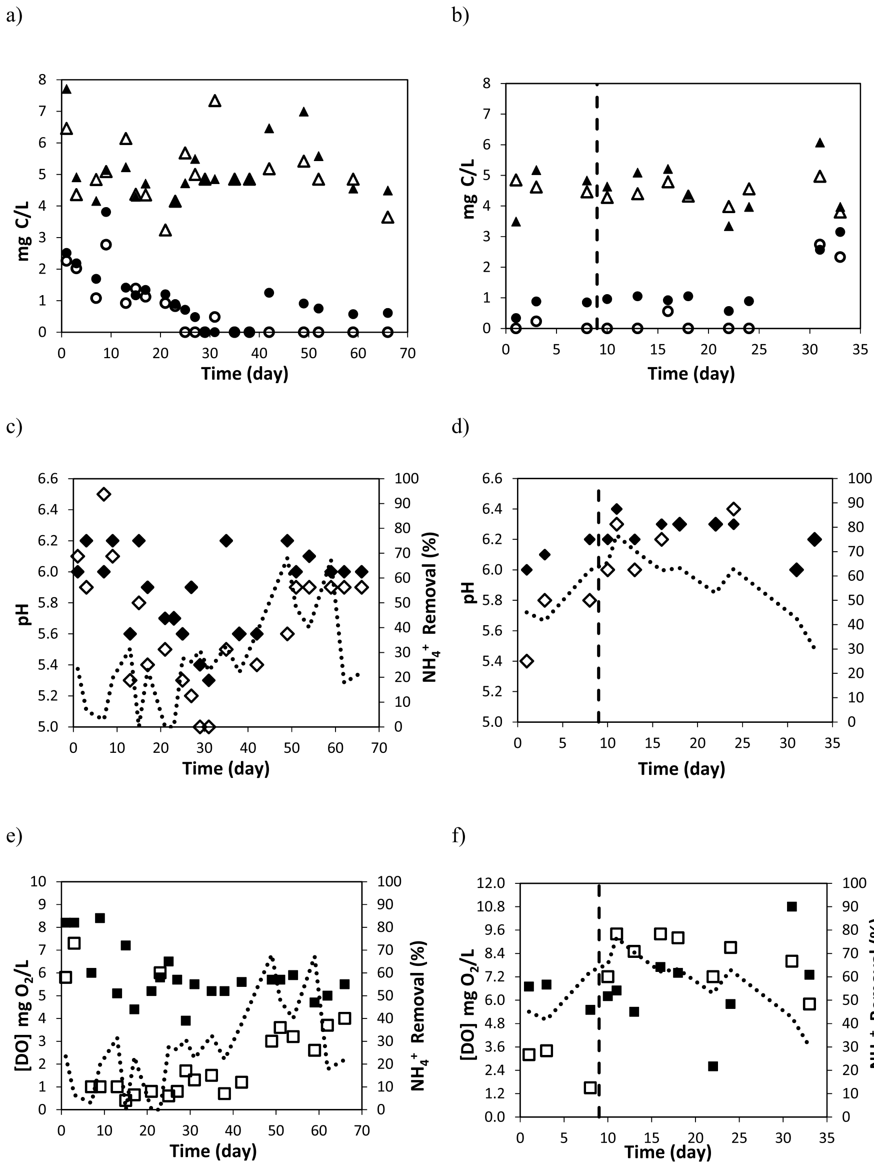

Fig. 2. TOC influent (▲) and effluent (Δ) concentrations and IC influent (●) and effluent (○) concentrations on the Aquammox (a) and AquaMab CFGRs (b). The pH at the influent (◆) and effluent (◇) of the Aquammox (c) and AquaMab CFGRs (d). The DO concentrations at the influent (■) and effluent (□) of the Aquammox (e) and AquaMab CFGRs (f). The dotted lines (···) in figures c–f represent the ammonium removal. The vertical dashed line in figures b, d and f splits the periods before and after microalgae bioaugmentation.

3.3. Dissolved oxygen concentration profile

The DO concentration in the influent of both reactors was similar to that of the water from the facility's fishponds, ranging from 3.9 to 8.2 and 2.6–10.8 mg O_2/L in the Aquammox and the AquaMab reactors, respectively (Fig. 2e and f). In the Aquammox reactor, throughout all the operational period, the DO content at the effluent was lower than that at the reactor entrance. Until day 42 this difference was substantially high, with the DO concentration in the effluent reaching levels below 1 mg O_2/L (Fig. 2e). Afterwards, the DO effluent concentrations increased approximately 3.4 mg O_2/L. On the other hand, in the AquaMab CFGR, two distinct DO concentration profiles were observed. In the first days of operation, the DO concentration decreased about 55–81% during the treatment process, with DO effluent concentrations of 1.5–3.4 mg O_2/L (Fig. 2f). After microalgae addition on day 8, an inversion of the DO profile was observed, with DO effluent concentrations higher than those in the influent. After microalgae bioaugmentation, the AquaMab CFGR presented DO effluent concentrations of up to 9.4 mg O_2/L.

3.4. Biomass metabolic activities

The inoculum of the Aquammox CFGR presented a SAA of 352 ± 48 mg N_2–N/(g VSS·d), although this anammox activity disappeared completely over operation. On day 66, the reactor biomass presented a SAA of 0 mg N_2–N/(g VSS·d).

The AGS inoculum of the AquaMab CFGR, which consisted of biomass from a pilot-scale CFGR treating aquaculture streams, presented a SDA of 51 ± 4 mg N_2–N/(g VSS·d). After 12 days of the bioaugmentation with the microalgae consortium (day 20), the SDA of the biomass disappeared completely.

Respirometric assays were carried out to determine the maximum AOB activity and the photosynthetic activity of the Microalgae-Bacteria biomass developed in the AquaMab CFGR. Fig. 3 depicts the respirogram chart obtained for the biomass collected on day 32. The biomass endogenous activity (in dark conditions and without any substrate) was of 127.9 mg O_2/(g VSS·d). When light was provided, the maximum oxygenation activity of the microalgae present in the biomass was 372 mg O_2/(g VSS·d). The biomass AOB activity was determined through the addition of ammonium, in dark conditions, and was of 47.9 mg NH_4^+-N/

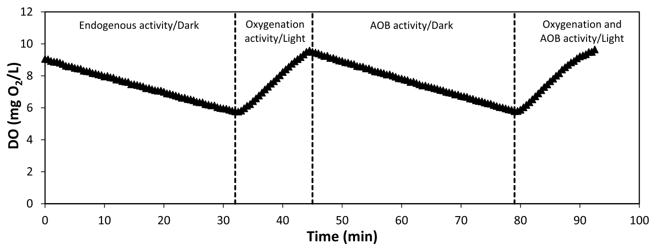

Fig. 3. Profile of the DO concentration (▲) during a respirometric assay with the AquaMab biomass collected from the reactor on day 32.

(g VSS·d). In addition, in the presence of light, the oxygenation capacity of the Microalgae-Bacteria biomass when the AOB activity is co-occurring was of 239.4 mg O_2/(g VSS·d).

3.4. Biomass performance

In the Aquammox CFGR, mature granules collected from a full-scale PN-AMX ELAN® reactor were used as inoculum, whereas for the AquaMab CFGR, the inoculum was the AGS from a pilot-scale CFGR treating the extremely low-strength wastewater of the same farm (Santorio et al., 2022).

In the Aquammox CFGR, the initial granules had an intense red appearance with a dense core and an average diameter of 1.37 mm (Fig. 4a). After 34 days facing the extremely low-strength conditions of the aquaculture effluent, the PN-AMX granules lost the intense red colour and dark zones appeared on their surface (Fig. 4b and S1a). Afterwards, the granules started to lose their integrity and by the end of reactor operation, the biomass had a dark brown colour and a flocculent appearance (Fig. 4c).

In the AquaMab reactor, the initial AGS granules had a brown colour and an average diameter of 0.36 mm (Fig. 4d). The microalgae inoculum had a flocculent green appearance (Fig. 4e). After the microalgae addition, the brown granules started to acquire a green colour shade and, throughout operation, the green colour tone of most of the granule surface was intensified to dark green (Fig. 4f and S1b). Five days after the microalgae bioaugmentation, the average granules size diameter decreased to 0.31 mm (day 13) but, over time, a tendency for granules to increase in size was observed, and on day 25 their average diameter was 0.46 mm (Fig. 4f).

An improvement of the biomass settling properties was observed throughout the AquaMab operation. The AGS inoculum presented a SVI_{30} of 105 mL/g TSS and a SVI_{30}/SVI_5 ratio of 0.54. Four days after microalgae addition (day 12), the SVI_{30} decreased to 67 mL/g TSS and the SVI_{30}/SVI_5 ratio improved to 0.90, indicating that the biomass required 5 min to almost entirely complete its settling. This improvement of the granules settling properties continued until the end of the experiment with SVI values between 62 and 75 mL/g TSS and SVI_{30}/SVI_5 ratios between 0.82 and 1.00 (Fig. 5a). The settling velocity followed a similar decreasing behaviour. On day 3, the biomass presented a settling velocity of 3.59. After 5 days of microalgae addition (day 13), this velocity increased to 6.03 m/h. Onwards the granules settling velocities ranged between 5.84 and 6.24 m/h (Fig. 5b). Moreover, the physical properties of the resulting mixed Microalgae-Bacteria granules that were developed improved throughout reactor operation. The granule diameter increased from 0.36 to 0.46 mm, probably due to the formation of a new algae external layer. Additionally, the SVI_{30} and the settling velocity of the developed granules also improved from 105 to 62 mL/g TSS and from 3.59 to 6.24 m/h, respectively. This settling improvement led to an effluent quality improvement since the effluent solids concentration decreased from 6.0 mg TSS/L before the microalgae addition to 3.7 mg TSS/L after microalgae bioaugmentation.

The AquaMab CFGR was able to retain the biomass inside the system. The initial AGS biomass concentration was 0.56 g TSS/L. On day 8, a suspended microalgae inoculum with a concentration of 0.15 g TSS/L was used to bioaugment the reactor. Four days after microalgae addition, the biomass concentration increased to 0.74 g TSS/L, and a tendency to progressively increase was observed, achieving a maximum biomass solids content of 1.27 g TSS/L, on day 25. On the last operational days, the biomass solids concentration in the reactor dropped to

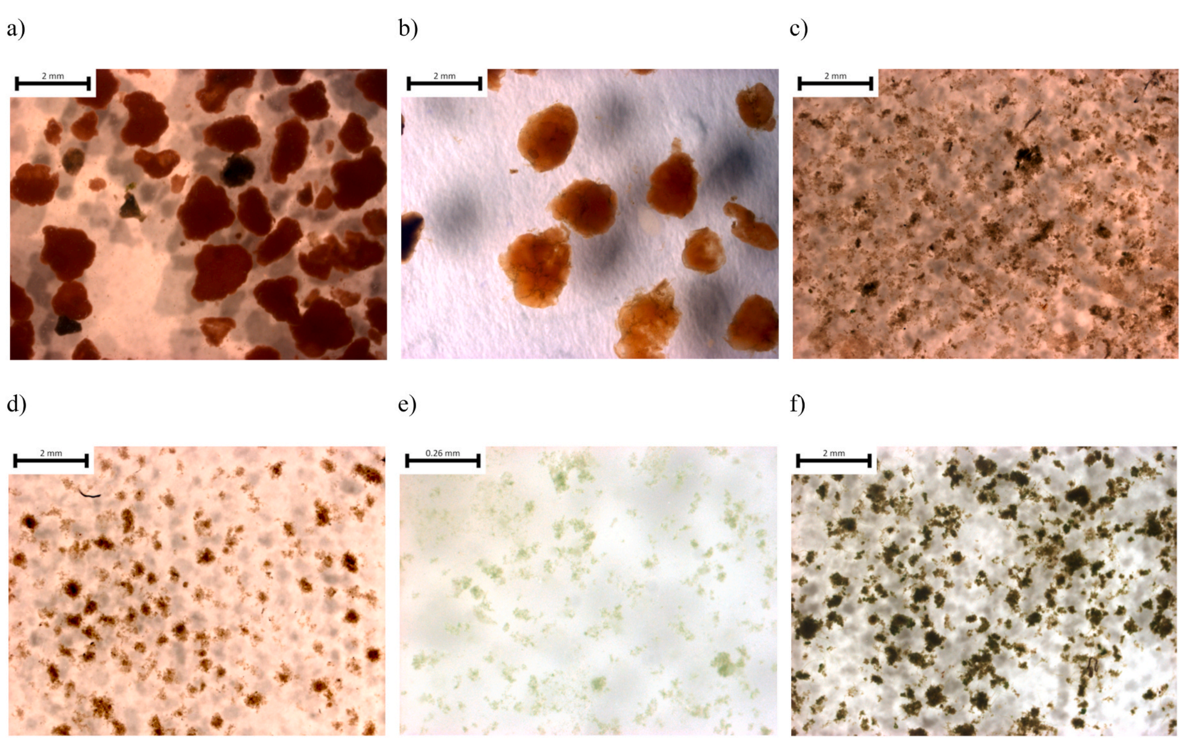

Fig. 4. Aquammox CFGR biomass on days 0 (a), 34 (b) and 66 (c) of operation and the microalgae inoculum (e), and AquaMab CFGR biomass on days 0 (AGS inoculum) (d) and 18 (10 days after bioaugmentation) (f). The size bars indicate 2 mm (a, b, c, d and f) and 0.26 mm (e).

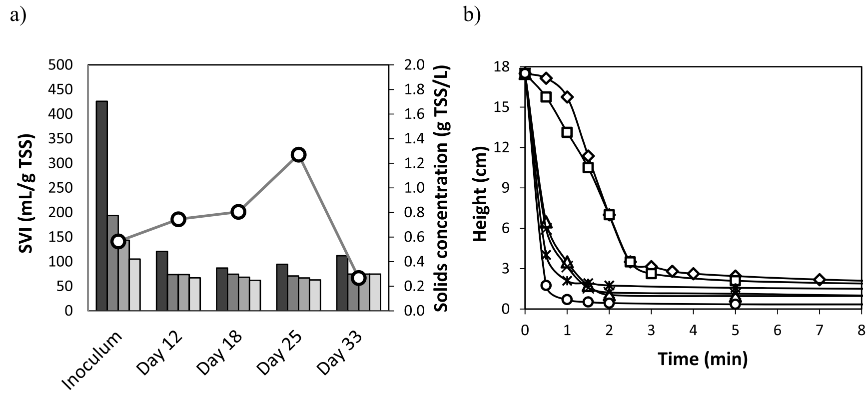

Fig. 5. Evolution of the AquaMab biomass properties: a) Sludge volume index values at minute 1 (■),5 (■),10 (■) and 30 (■) and the reactor biomass concentration (○) throughout the reactor operation; b) Biomass settling velocity curves on days 1 (◊),3 (□),13 (Δ),18 (x), 25 (Ж) and 33 (○).

0.27 g TSS/L, probably due to the high decrease of influent temperature under 12 °C.

4. Discussion

4.1. Effect of extremely low-strength aquaculture water streams in PN-AMX processes

During the Aquammox CFGR operation, nitrification was the main process taking place and the biomass was able to oxidize up to 68 and 100% of the ammonium and nitrite fed, respectively. Total nitrogen removal did not occur. In fact, the observed ammonium removal rate (ARR) was 18.4 mg NH_4^+-N/(g VSS·d). This relatively high AOB activity was already present in the inoculum while the NOB activity, absent in the inoculum, developed throughout the Aquammox system operation. The high DO concentrations in the influent could have promoted the proliferation of NOB species. From day 42 onwards, the improvement of the consumption of IC coincided with the improvement of the nitrification process, probably because nitrifying bacteria are autotrophic microorganisms (Henze et al., 2008). At the beginning of reactor operation, the SAA biomass activity was 352 mg N_2–N/(g VSS·d), a typical activity of PN-AMX granules, although, over time, it declined being completely absent on day 66. In addition, the nitrogen mass balance showed that the sum of the ammonium and nitrite removed from the wastewater corresponded to the nitrate produced and accumulated in the reactor effluent. The lack of anammox activity and the development of NOB activity justified the Aquammox biomass performance in terms of nitrogen removal. Also, the characteristic red colour of anammox biomass, caused by the presence of heme *c* proteins (Cytochrome *c* co-enzymes) and the SAA (Kang et al., 2018) present in the inoculum, progressively disappeared over reactor operation and, consequently, the biomass turned into dark brown granules. Furthermore, since anammox biomass metabolism causes basification, the lower pH value in the effluent compared to that in the influent also emphasizes the low anammox activity. The TOC concentration in the effluent, higher than those in the influent in many operational days, could presumably be due to biomass decay. By the end of operation, the granules completely disaggregated to flocculent biomass, perhaps because this biomass could not adapt to the aquaculture's extremely low-strength water conditions. Overall, this poor biomass performance resulted in a highly variable ammonium and organic matter removal performance, showing that the PN-AMX process is not an adequate technology to be applied in RAS, under extremely low-strength water conditions.

The high DO influent concentration, together with the extremely low ammonium and nitrite concentrations in the water streams produced at the facility, are probably the main factors leading to the poor performance of the Aquammox reactor. The DO concentration in the influent was near to saturation (around 8 mg O_2/L) and,as such, oxygen can fully penetrate the granules (Morales et al., 2015). Moreover, the low ammonium and nitrite concentrations in the wastewater can favour the oxygen diffusion into the granule core, due to its low consumption, thus leading to the loss of the anoxic conditions necessary for the growth of the anammox bacteria. Besides, the PN-AMX granules were collected from the effluent of an anaerobic digester treatment. The ammonium concentration in such kind of effluents is usually around 540–1045 mg NH_4^+-N/L (Morales et al., 2015), which is much higher than that of the extremely low-strength influent used in this study. Other parameter to consider that can affect the correct performance of anammox bacteria activity is the temperature, as the PN-AMX inoculum come from a reactor working at temperature around 30 °C, while in the Aquammox reactor the anammox granules were exposed to values of 17 °C. These drastic changes (DO and nitrogen concentrations and temperature) could have severely affected the granular biomass, which was not able to adapt to such conditions in a short period of time, especially considering the slow grow of anammox bacteria with reported doubling times around 11 days.

The FA concentrations in the influent ranged between 0.1 and 0.6 μg NH_3–N/L, being much lower than the inhibition values for anammox bacteria (20–40 mg NH_3–N/L) (Jin et al., 2012). However, the low influent pH in these water streams may have displaced the nitrite equilibrium to high FNA levels, even at the low nitrite concentrations observed. As a result, the influent FNA concentrations in the Aquammox CFGR were between 0.1 and 1.9 μg HNO_2–N/L. Fernández et al. (2012) reported that FNA concentrations over 1.5 μg HNO_2/L caused a decline of the nitrogen removal efficiency and a destabilization of the anammox process. Therefore, the low pH, and consequently, the high FNA concentrations in the aquaculture streams, could have affected the anammox biomass activity. Additionally, according to Strous et al. (1999), the ammonium and nitrite affinity constants for anammox processes are below 0.15 mg NH_4^+-N/L and 0.05 mg NO_2^--N/L. Thus, the ammonium and nitrite concentrations in the aquaculture streams were high enough for the Anammox process to occur at its maximum rate.

Therefore, although the inoculum presented PN activity to oxidize half of the ammonium to nitrite aerobically as well as anammox activity to oxidize the remaining ammonium and the produced nitrite to nitrogen gas anaerobically, after being exposed to the freshwater from the aquaculture, these processes lost their dominance in the biomass. The biomass microbial populations evolved in such a way that complete nitrification started to occur, leading the complete oxidation of ammonium to nitrate. These changes in microbial populations were probably imposed by the low influent nitrogen concentrations and the relatively high DO concentrations which could have promoted the development of NOB species (aerobic) and hindered the activity of AMX bacteria (anoxic and sensitive to DO presence).

As far as the authors know, no research study has been reported for the treatment of such extremely low nitrogen concentrations in freshwater recirculating wastewater with a PN-AMX system. van Kessel et al. (2010), through mass balance calculations, inferred the presence of anammox activity in moving bed biofilters treating freshwater from RAS but, no anammox strains were identified in the biomass by 16 S DNA massive sequencing analysis. Thus, in the present study, the Aquammox CFGR performance showed that the PN-AMX granular biomass could hardly adapt to treat recirculating aquaculture effluents. Since the nitrification processes (nitritation and nitratation) took place in the reactor, the chosen strategy to remove nitrogen along with reduced oxygen consumption via PN-AMX processes was not successful.

4.2. AGS bioaugmentation with microalgae to treat extremely low-strength aquaculture water

A successful bioaugmentation of the AGS with microalgae was achieved despite the low nutrients and carbon concentrations in the aquaculture water streams, the short HRT applied during reactor operation, and the poor settling properties of the suspended microalgae consortium. The adopted strategy of mixing the cultures without feeding the reactor (12 h) and gradually shortening the HRT throughout the first days after microalgae addition, probably allowed for the microalgae to attach to the existent granules, that rapidly acquired a green colour shade in the course of only one day. Over reactor operation, the granules green colour intensification indicated that the microalgae culture successfully colonized the granule surface and proliferated.

After microalgae addition, no major changes were observed in the ammonium and nitrite removal efficiencies, which reached up to 77% and 80% of removal, respectively. However, after microalgae addition, the AquaMab reactor improved nitrate removal performance. Fan et al. (2021) applied Microalgae-Bacteria granular sludge to treat aquaculture effluents achieving an ammonium removal of 85%. Nevertheless, that reactor was fed with synthetic media that contained higher carbon and nutrients concentrations (280 mg COD/L, 11.4 mg NH_4^+-N/L and 9.9 mg NO_2^--N/L), was operated in SBR operational mode, applied longer HRTs (8 h) and the applied inoculum was composed of Microalgae-Bacteria granules previously cultivated. Besides, Ji et al. (2022) treated aquaculture streams in a non-aerated continuous-flow tubular reactor with a mixed Microalgae-Bacteria biomass. However, that study was conducted at HRTs of 6 h, using a higher concentrated synthetic medium (11.4 mg/L NH_4^+-N, and 9.9 mg/L NO_2^--N) as feeding, and applied an inoculum composed of mature Microalgae-Bacteria granules.

Thus, the present study was the first where AGS was bioaugmented successfully with a suspended microalgae consortium in a continuous reactor facing *in-situ* aquaculture effluents, which physical chemical characteristics slightly varied over time depending on rearing and weather conditions. Previous studies indicate that when the aquaculture treatment is only based on microalgae cultures, it is often necessary to perform at long HRT (24 h) to achieve a proper nutrient removal performance (Liu et al., 2019). Nevertheless, in the present study, the microalgae-bacterial biomass performed properly at an extremely short HRT of approximately 30 min. This benefited the separation of the biomass from the treated effluent, as already reported by Quijano et al. (2017). Moreover, the settling velocity improvement showed that the AquaMab CFGR can perform at shorter HRT after microalgae bioaugmentation, being able to retain microalgae within the system performing at high influent flow. Ahmad et al. (2019) attempted Microalgae-Bacteria granular sludge to treat medium-strength synthetic wastewater in a continuous reactor, without aeration. Unlike the present study, a drop in the TN removal performance was observed after stopping the aeration and the recovery of the system performance was only achieved by applying intermittent aeration.

Some authors suggested that the DO generated by microalgae through photosynthesis could be inadequate for bacteria to use (Ahmad et al., 2017; Tang et al., 2016; Zhang et al., 2018). However, in the present study, the oxygen produced by microalgae seemed to be used by bacteria since the reactor's performance,concerning nitrogen removal and biomass settling properties, slightly improved after microalgae bioaugmentation.

After the formation of the mixed Microalgae-Bacteria granules, nitrate removal started to occur. Before the microalgae addition in the AquaMab reactor, the nitrate content production corresponded to the consumption of the ammonium and nitrite contents in the influent. Santorio et al. (2022) reported the same behaviour in the pilot-scale CFGR from where the inoculum for the AquaMab CFGR was taken. However, after microalgae addition, the mass balances were not justified by the nitrifying activity. The amount of nitrate produced by the system was lower than the amount of ammonium plus nitrite in the wastewater that was available for oxidation or sometimes the nitrate concentrations in the effluent were lower than those in the influent. Since the SDA completely declined after bioaugmentation, the nitrate consumption can hardly be due to the denitrification process. Thus, nitrate consumption should be mainly due to microalgae growth as it is one of the nitrogen forms that these microorganisms can use as the nitrogen source (De-Bashan et al., 2004). In addition, the SDA loss could be related with the excess of oxygen in the system due to the microalgae photosynthetic activity, thus causing higher oxygen levels available that can diffuse through the inner core of the granules. The evolution of the pH profile also supports this hypothesis. Before microalgae addition, the pH of the reactor's effluent was lower than the one of the influent, indicating the occurrence of nitrifying metabolism during the treatment process. However, after microalgae addition, either the pH of the influent and effluent was similar or was higher at the effluent. This indicates that the microalgae consumed the dissolved carbon dioxide available, causing water basification.

The nitrate consumption capacity of the AquaMab biomass could avoid the accumulation of this nitrogen form in a long-term RAS process. This hypothesis could represent positive effects, for instance, a decreased nitrate concentration in the water to be discharged into the nearby water bodies and coasts, minimizing potential environmental problems such as eutrophication. Additionally, the potential excess of microalgae biomass produced inside the reactor could be valorised as a high added-value product. Furthermore, as the microalgae are growing in the form of granules this could turn their separation from the liquid fraction easier (Zhang et al., 2021).

Finally, one of the most important aspects of AquaMab for its application as a RAS treatment process is the production of an effluent with high DO levels. The presence of microalgae within the biomass led to an increase of the DO concentration in the effluent compared to that of the influent, while maintaining high ammonium and nitrite removal performances. On some operational days, this DO concentration in the effluent reached oxygen saturation levels of 9.4 mg O_2/L, which surpass the requirements for rainbow trout of 6–8 mg O_2/L (Ebeling and Timmons, 2012). The respirometric test showed that the DO concentration increased due to microalgae activity simultaneously to bacterial nitrification.

In the AquaMab system, before bioaugmentation, the inoculum had the capacity to oxidize the ammonium to nitrate aerobically via nitrification with the consequent accumulation of nitrate and DO concentration depletion. After bioaugmentation with microalgae, the bacteria and the added microalgae probably developed a symbiotic relationship in such a way that complete nitrification occurred while the produced nitrate was readily consumed and oxygen was produced. Then, the microalgae populations within bacterial granules consumed the nitrate available (avoiding nitrate accumulation) for cellular growth and produced oxygen (increasing the DO concentration of the media) by photosynthetic processes. Furthermore, the retention of the microalgae within the system at the short applied HRTs was only possible due to their capacity to attach to the AGS surface.

Considering that the aquaculture farms that recirculate water (such as Grupo Tres Mares) must oxygenate the water of the ponds with liquid oxygen or aerating devices, this treatment system could reduce the oxygenation costs while concomitantly reducing the ammonium and nitrite concentrations in the water which are target pollutants to be removed if water recirculation for the ponds is aimed for. Thus, the strategy of bioaugmenting AGS with microalgae not only reduces the oxygen requirements of the treatment process but also reduces the need of water oxygenation for reuse. The potential use of solar light instead of LED devices should be studied in future research, in order to diminish the energy costs of this technology, validating its application at larger scales. Other aspect to consider in the future research work is to study the possible effect of microalgal toxins over the fishes, although the use of an inoculum of microalgae collected from the fishponds is expected to reduce this negative effect.

4.3. Comparison between different CFGRs treating aquaculture extremely low-strength streams

The performances of the Aquammox and the AquaMab CFGRs are the continuation of the study carried out by Santorio et al. (2022). In the mentioned study, the granulation in a CFGR successfully occurred treating extremely low-strength freshwater aquaculture effluents with a high nitrifying activity. The experiments with the three CFGRs (Table 2) were performed in the same rainbow trout farm and at the same dry season. However, it is necessary to point out that AquaMab was inoculated with biomass from the pilot plant CFGR, already adapted to the fish farm effluent conditions, while the Aquammox was inoculated with biomass treating the effluent from an anaerobic sludge digester, characterized by higher nitrogen concentrations and temperature. The best ammonium and nitrite removal performances were achieved in the pilot-scale CFGR (Table 2). However, the three reactors provided an effluent with quality, good enough for recirculation, in terms of ammonium and nitrite concentrations, which are below the toxic limits for rainbow trout: 0.78 mg NH_4^+-N/L (Liao and Mayo, 1972) and 0.06 mg NO_2^--N/L (Russo et al., 1974). Nevertheless, the AquaMab CFGR seemed to be the only technology able to avoid the nitrate accumulation in the water to be recirculated (Table 2). Besides, from the three reactor processes, it is the only one with the capacity to produce effluents with adequate oxygen levels for further water reuse in the fish ponds, thus reducing the oxygenation requirements.

5. Conclusions

Two technologies based on PN-AMX biomass (Aquammox CFGR) and Microalgae-Bacteria biomass (AquaMab CFGR) were studied as alternatives to reduce the oxygen requirements to treat extremely low-strength aquaculture streams (0.3–1.4 mg N/L and 3.3–7.7 mg C/L) at very short HRT (14–86 min).

The Aquammox CFGR was not adequate to treat freshwater aquaculture streams for reuse, as the oxygen availability in the treated effluent did not meet the water quality requirements for further recirculation for the trout tanks. Although high ammonium and nitrite removal percentages were reached (68 and 100% respectively), the DO concentration in the effluent was too low, below 1 mg O_2/L, insufficient for fish health and survival. Moreover, the initial PN-AMX activity of the biomass could not be maintained in such conditions and biomass SAA disappeared completely. Although complete nitrification occurred, the granules lost their integrity and their red distinctive colour.

On the contrary, the AquaMab CFGR performed excellent in terms of DO availability in the treated effluent for recirculation, simultaneously allowing for a reduction of ammonium and nitrite concentrations below toxic limits for rainbow trout. The mixed Microalgae-Bacteria biomass developed in the reactor allowed the production of enough oxygen for the nitrification process to occur (ammonium and nitrite removals up to 77 and 80%, respectively) and even to maintain the effluent DO concentration near to saturation values (8–9 mg O_2/L). Besides, fast microalgae attachment to bacterial granules took place in less than 5 days, which was accompanied by an improvement of the biomass settling properties that reached a SVI_{30} value of 62 mL/g TSS and a settling velocity of 6.24 m/h.

Table 2
Performance of the different CFGRs treating freshwater aquaculture recirculation streams.

	Aquammox CFGR	AquaMab CFGR	Pilot plant CFGR[a]
Type of biomass	PN-AMX	Microalgae-Bacteria	AGS
NH_4^+-N Removal (%)	68	77	81
NO_2^--N Removal (%)	100	80	100
NO_3^--N Accumulation	Yes	No	Yes
Effluent DO concentration (mg O_2/L)	0.5–3.5	7.2–9.4	0.0–2.3

[a] According to Santorio et al. (2022).

Authorship contributions

Conceptualization: Catarina L. Amorim, Ángeles Val del Río and Luz Arregui., Methodology: Ángeles Val del Río., Formal analysis: Sergio Santorio, Investigation: Sergio Santorio, Validation: Ana T. Couto, Catarina L. Amorim, Ángeles Val del Río, Anuska Mosquera-Corral and Paula M. L. Castro., Writing - Original Draft: Sergio Santorio, Writing - Review & Editing: Ana T. Couto, Ángeles Val del Río Anuska Mosquera-Corral and Paula M. L. Castro., Visualization: Catarina L. Amorim, Ángeles Val del Río, Anuska Mosquera-Corral and Paula M. L. Castro., Supervision: Luz Arregui, Anuska Mosquera-Corral and Paula M. L. Castro., Project administration: Luz Arregui, Anuska Mosquera-Corral and Paula M. L. Castro., Funding acquisition: Luz Arregui, Anuska Mosquera-Corral and Paula M. L. Castro.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.136184.

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