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D.2.2 – Integration salmon and microalgae cultivation in a coupled RAS-IMTA system

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TABLE OF CONTENT

List of Figures

List of Tables

List of Acronyms

Abbreviation / Acronym	Description
BW	Brackish water
Cs	Chlorella sorokiniana
DOC	Dissolved organic carbon
DW	Dry weight
FW	Freshwater
N	Nitrogen
No	Nannochloropsis oculata
NO_3	Nitrate
P	Phosphorous
PBR	Photobioreactor
PO_4	Phosphate
Pt	Phaeodactylum tricornutum
RAS	recirculating aquaculture systems
RAS-IN	RAS water going into the reactor
RASw	RAS water
SW	saltwater

Executive Summary

Using microalgae for the remediation of the nutrients in the effluent water from recirculating aquaculture systems (RAS) can be a promising alternative to current water treatment techniques and offers an opportunity to reduce nutrient losses and transform a waste stream into valuable products (Ende et al., 2024). The microalgal biomass can be valorized into a sustainable feedstock for a variety of commercial products such as feed or food (Villanova et al., 2023).

In D2.2, the feasibility of the integrated RAS-microalgae unit at pilot-scale is tested and demonstrated through four microalgal cultivation experiments with three different microalgal species; *Chlorella sorokiniana, Phaeodactylum tricornutum* and *Nannochloropsis oculata*. Microalgae cultivation was performed in perfusion mode alongside the fish, by continuously feeding filtered RAS effluent water into the bioreactor, while nutrient depleted water was continuously removed, and the microalgal cells retained in the reactor through membrane filtration. Biomass productivity of microalgae and nutrient removal capacities were assessed and the impact of the RAS water on the microalgal biomass quality will be investigated.

1. Introduction

The main objective of the INNOAQUA project is to pave the path towards the upcoming sustainable and diversified EU in-land aquaculture industry by leaning on the demonstration and mainstreaming of innovative algae-based foods and solutions and ecology, circularity and digitalization concepts.

Land-based aquaculture systems offer significant advantages over coastal cage culture, such as improved management with the capacity to predict the outputs of production cycles, the capability to mitigate environmental interactions (escapes, infectious diseases, parasites, etc.) and the potential to be sited "closer-to-market", delivering fresh seafood with less transportation time and impact to the consumers. Recirculating aquaculture systems (RAS) systems and land-based integrated multi-trophic aquaculture (IMTA) approaches have been widely regarded as game-changers for the aquaculture industry due to their potential to reduce the environmental impacts and to increase the economic returns as compared with other conventional monoculture production systems. Nonetheless, studies combining IMTA and RAS are very limited, specially at relevant scale conditions. Several reasons could be pointed to for this situation: (i) the lack of knowledge in a fish-farm to manage production systems for other organisms; (ii) the lack of confidence that the IMTA water can be safely re-used by the fish; (iii) the lack of suppliers of microalgae/seaweed "seed", just as it exists for animals and; (iv) the absence of a clear market knowledge for other than fish. Thus, further research is required to demonstrate with evidence the benefits of RAS-IMTA systems at relevant scale to accelerate and incentivize its commercial implementation.

INNOAQUA will contribute to this regard by demonstrating the environmental and socio-economic benefits of integrating fish and algae cultivation at two DEMO# sites, using coupled RAS-IMTA systems and aligning culture optimization with waste management. **DEMO#1** will demonstrate the integration of **RAS Atlantic salmon aquaculture with microalgae cultivation.**

1.1 Purpose of the document

In the original call from which the INNOAQUA project received funding (HORIZON-CL6-2022-FARM2FORK-02-05), the Commission called for innovative solutions to environmentally friendly, inclusive, safe and healthy seafood production through innovation in the supply chain, through implementation of the ecosystem approach for sustainable management of marine or freshwater fisheries or in aquaculture development.

Work package 2 of INNOAQUA (Sustainable Aquaculture Practices) has five main objectives:

I. the implementation of an ecosystem approach in two demo sites.
II. the demonstration of coupled RAS and IMTA systems for an improved environmental sustainability of aquaculture.
III. the production of algae biomass for new seafood and packaging materials development in the project.
IV. the development of digital solutions for aquaculture.
V. the development of aquaculture sludge valorisation strategies.

This deliverable shows the feasibility of an integrated cultivation of salmon and microalgae at demonstration scale by implementing a coupled RAS-microalgal unit and assessing microalgal biomass productivities and nutrient removal capacities.

1.2 Structure of the document

The document is divided into 3 sections. Section 1 summarizes the methodologies used to set up and test the integrated Ras-microalgae unit. Section 2 shows the results of the cultivation experiments and section 4 gives a summarizing conclusion.

1.3 Relation to other project deliverables

This document is connected to Deliverable 3.1 *Characterization results of algae biomass and fish/seafood processing waste*, where two different strains of the three microalga species *C. sorokiniana, P. tricornutum* and *N. oculata* were tested for their biomass composition and growth rates. From each species 1 strain was selected for further experiments and upscaling. Those selected strains were used in D.2.2. It is also connected to D.2.1 *Aligning INNOAQUA's Sustainable Aquaculture Practices with the EEA,* that showed how the approach and demonstrations developed within INNOAQUA fit well within the Ecosystem Approach to Aquaculture (EAA) framework and assessed the legal and regulatory bottlenecks to wide scale deployment of IMTA in Europe. Moreover, it will be used in the deliverables connected to WP5, for the sustainability and circularity analyses.

2. Methodology

2.1 Implementation of the integrated RAS-microalgal unit

2.1.1 Ras system

Recirculating aquaculture systems (RAS) are closed, land-based fish production systems, that allow a great degree of water recirculation and reuse by either removing or converting fish waste products (Ende et al., 2024; Villanova et al., 2023). The waste products from the fish are solid sludge (from feces and feed leftovers) and dissolved ammonium. The rearing water from the fish tanks flows through different treatment steps where first, the fish sludge is being removed by mechanical filters, and then the ammonium in the water is being converted to less toxic nitrate in a biofilter, and the purified water is returned to the fish tanks. During the life cycle of salmon, the salinity of the water shifts from fresh to brackish and finally salt water.

The fish production system used for setting up the integrated RAS-microalgal unit was a 2500 L RAS module (Alpha Aqua A/S, Esbjerg, Denmark) at Marineholmen RASlab AS in Bergen, Norway (Fig. 1). The RAS module consisted of a fish tank (1), swirl separator (2), mechanical filter (3) and biofilter (4), and 5% of the water was exchanged daily. Two rounds of salmon production were performed for the experiment. For round 1, 515 Atlantic Salmon were cultivated from November 2nd, 2023, until February 29th, 2024, and the system switched from fresh to brackish water on December 12th 2023, and from brackish to seawater on January 25th 2024. For round 2, 200 fish were cultured from June 25th, 2024, until November 1st 2024. The system switched from fresh to brackish water on August 22nd and from brackish to seawater on October 7th.

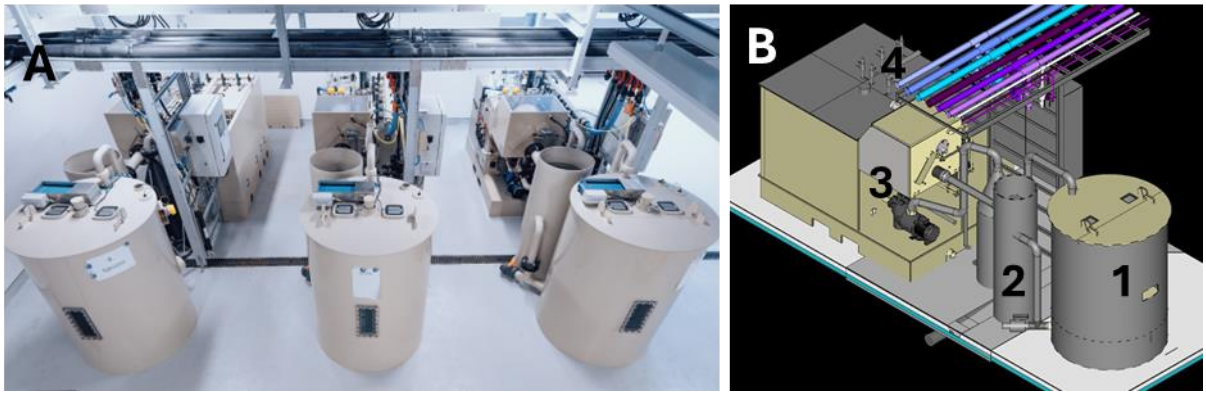

Fig. 1. Pilot-scale RAS-module (2500 L) at Marineholmen Ras-lab. A: Picture of three Ras-modules. B: schematic drawing of a RAS-module with (1) fish tank, (2) swirl separator, (3) mechanical filter and (4) biofilter). (Source: T- 41 - GC Rieber Eiendom)

2.1.2 Microalgal photobioreactor

The photobioreactor (PBR, Fig. 2) used for setting up the integrated RAS-microalgal unit was a 25 L tubular photobioreactor (GemTubeTM RD1-25). The reactor consisted of glass pipes (32 x 2.2 mm), connected to a collection vessel (2 L) and was equipped with a pH and temperature sensor. An air pump at the bottom of the reactor created an ingoing airstream, allowing culture mixing and circulation through the tubes. Carbon supply was ensured by pH-controlled injection of CO_2 into the ingoing airstream, and irradiance was provided by dimmable LED lights, on both sides of the reactor (maximum irradiance 600 µmol m^{-2} s^{-1}). Before cultivation, the PBR was chemically cleaned and sterilized with hypochlorite (NaOCl 5%).

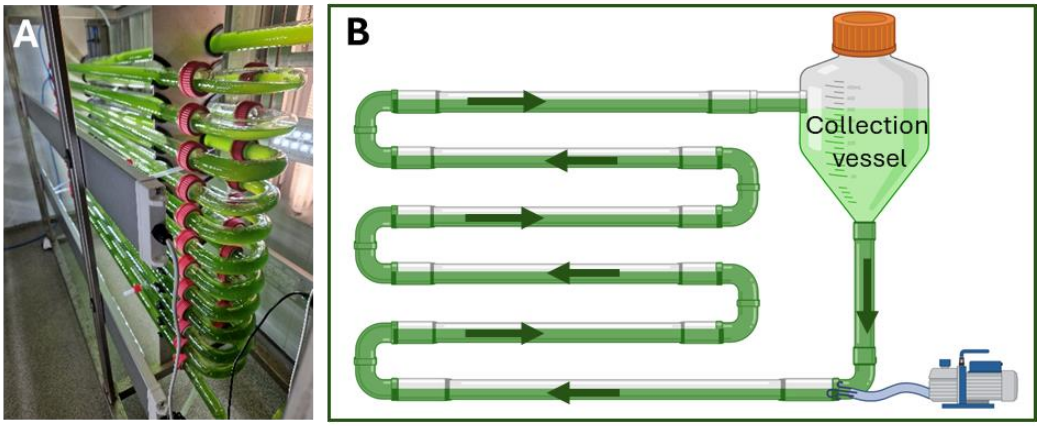

Fig. 2.Picture (A) and scheme (B) of the tubular photobioreactor (25 L) used for the integrated RAS-microalgae unit.

2.1.3 Nutrient supply in the integrated RAS-microalgal unit

Besides carbon, which is being supplied as CO_2 gas, the basic nutritional requirements for microalgal production are nitrogen (N), phosphorous (P), and additional micronutrients (µ). To produce 10 g of microalgae biomass, approximately 1 g of N, and 0.1 g of P are required (Acién Fernández et al., 2018). The water in RAS systems contains nitrate (NO_3), which is produced in the biofilter through nitrification from the ammonium released by the fish. The nitrate-nitrogen (NO_3-N) concentration of this "dirty water" is relatively low (<100 mg L^{-1}) but the total amounts, extrapolated to the water volume are significant. By using perfusion cultivation, the microalgal cells are retained in the PBR by membrane filtration. Therewith, large quantities of nutrient rich RAS effluent water (RASw) can continuously flow through the culture while nutrient depleted water can continuously be removed. This allows providing greater amounts of nitrogen to the microalgae and thus ensuring high biomass concentrations and productivities in the reactor, while at the same time large volumes of RAS effluent water can be treated. The NO_3-N present in the RAS effluent water was the sole nitrogen source used for microalgal cultivation. The P concentration in the RAS effluent water was lower than required (N:P > 20) for microalgae production and was therefore supplemented as PO_4, to reach an N:P ratio of 5 in the RAS water going into the reactor, which is the same N:P ratio as in the standard growth medium. The concentration of the required micronutrients (µ) in the RAS effluent water was unknown, therefore, micronutrients were also supplemented with the same N:µ ration as in our regular growth medium to avoid micronutrient limitation during cultivation (nutrient composition and concentrations for each species are described in 2.2.1)

2.1.4 Concept of the integrated RAS-microalgal unit

The microalgal PBR was placed next to the RAS-module at Marineholmen RAS-lab (Fig. 3). For perfusion cultivation of the microalgal cultures, effluent water from the RAS unit was pumped (P1) continuously via a filtration cascade consisting of 4 filtration units (VF-1, A-collection) with replaceable filter cartridges (pore sizes 20, 10, 1 and 0.2 µm) into the inlet at the lower part of the PBR. For phosphorous and micronutrient supplementation, a 50 L bucket was filled up with RAS effluent water, and P and µ were added to the tank (in concentrations according to

the provided nitrate concentration and flow rate). This spiked RAS effluent water (RASw-P-µ) was pumped (P2) from the bucket into the ingoing RAS effluent water stream (RASw-IN). The RASw then flowed together with the microalgal cells through the tubes of the bioreactor, where the nutrients were being consumed by the microalgae. To remove the nutrient depleted water from the microalgal culture, the culture was continuously pumped (PV1) from the collection tank at the top of the reactor, into the filtration device (Vibro® technology, 800kDA membrane; SANI Membranes, Denmark), where the culture was separated into two streams; a cell-free permeate, and a retentate containing the microalgal cells. The retentate was continuously pumped (PV2) back into the reactor at the bottom inlet while the permeate (P3) was discharged. To maintain a constant biomass concentration in the reactors, microalgal culture was continuously harvested (P4) with pump rates according to the microalgal growth rate. The pump rates of the different pumps were set individually for each microalgae species, to match nutrient concentrations in the RAS effluent water and growth rates of the algae. Total flow rates into the reactor and out of the reactor were equal (P1+P2 = P3+P4).

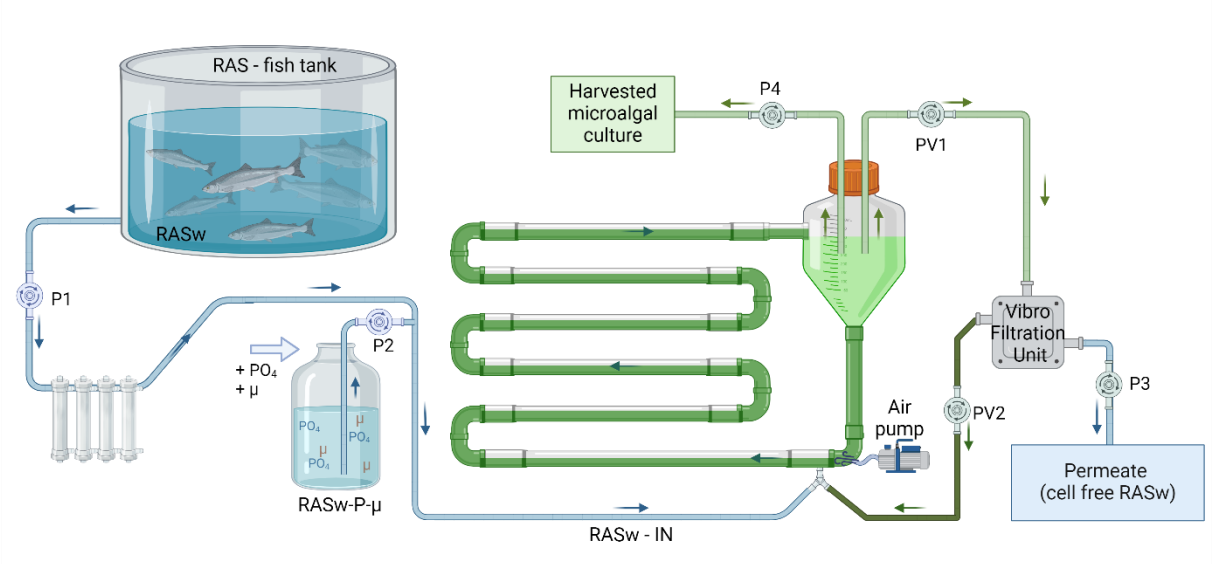

Fig. 3. Conceptual design of the integrated RAS-microalgae unit. RAS effluent water (RASw) from the fish tank, containing NO_3-N, was pumped continuously into the lower inlet of the reactor containing microalgae culture. Phosphate (P) and micronutrients (µ) were supplemented. From the collection tank at the top of the reactor, microalgal culture was pumped into the membrane filtration unit and from there back into the reactor. The filtered cell free RASw (permeate) was discharged. Microalgal culture was harvested with harvest rate = growth rate. Pump rates were: P1+P1=P1+P4. P1-P5= Pump 1-5. VP1-2= Pump to/from vibro filtration unit.

2.2 Microalgal perfusion cultivation experiments on RAS water

2.2.1 Microalgal species and inoculum production

Three microalgal species were used for cultivation experiments on integrated RAS-microalgae system; *Chlorella sorokiniana* (UTEX 1230)*, Phaeodactylum tricornutum* (B58, a Norwegian local strain) and *Nannochloropsis oculata* (NIVA-3/04). These three strains were selected in task 3.1 based on their biomass composition and growth rate. *C. sorokiniana* was used during freshwater phase of fish production, *P. tricornutum* during brackish (18 ppt salinity) and saltwater phase (32.5 ppt), and *N. oculata* during saltwater phase (32.5 ppt). Stock cultures were kept in 100 ml Erlenmeyer flasks. Inoculum for the 25 L reactor was produced in six 300 ml round-bottom bubble column cultures. For the inoculum production of *C. vulgaris* a modified 4N Bold Basal medium with following nutrient concentrations was used: 12.5 mM $NaNO_3$; 0.88 mM KH_2PO_4; 0.17 mM $CaCl_2{\cdot}2H_2O$; 0.3 mM $MgSO_4{\cdot}7H_2O$; 0.43 mM NaCl; 0.031 mM B; 0.002 mM Cu; 0.043 mM Fe; 0.017 mM Mn ; 0.001 mM Mo; 0.008 mM Zn; $2.96{\cdot}10^{-4}$ mM vitamin B1; $3.69{\cdot}10^{-7}$ mM vitamin B12; $2.05{\cdot}10^{-6}$ mM Biotin. *P. tricornutum* and *N.oculata* strains were grown in NORCE medium with following concentrations: 12.5 mM $NaNO_3$; 0.88 mM KH_2PO_4; 0.031 mM B; 0.002 mM Cu; 0.043 mM Fe; 0.017 mM Mn; 0.001 mM Mo; 0.008 mM Zn.

2.2.2 Cultivation experiments on RAS water

Two rounds of salmon production were performed in the designated RAS tank (Fig. 4). Round 1 (November 2023 – February 2024) was used to set up the integrated RAS-microalgal unit and to test microalgal perfusion cultivation with *P. tricornutum* during the saltwater phase (SW). During round 2 (July – October 2024) microalgal perfusion cultivation experiments were performed during each salinity phase (freshwater [FW], brackish water [BW], saltwater [SW]) (Fig. 4). For each salinity, a different microalgal species was used; *Chlorella sorokiniana* for freshwater (FW-Cs), *Phaeodactylum tricornutum* for brackish water (BW-Pt), and *Nannochloropsis oculata* for saltwater (SW-No).

Each cultivation was started with a batch cultivation followed by perfusion cultivation. The clean reactor was filled up with RAS effluent water from the RAS system (via filtration

cascade), and the nitrate-N and phosphate-P concentrations in the RAS water were measured. Based on the nitrate-N concentration and the anticipated biomass concentration during perfusion cultivation, additional nitrate, phosphate and micronutrients were added to the reactor. Microalgal inoculum was then added to the RAS-medium in the reactor, and the culture was allowed to grow until the desired biomass concentration was reached and nutrients in the reactor were used up (batch cultivation). The irradiance was increased progressively with increasing biomass concentration. Once the intended biomass concentration was reached (after 3-7 days) the perfusion cultivation was started. Two weeks of perfusion cultivation were planned for FW-Cs, BW-Pt and SW-No. However, due to technical problems with the Vibro filtration unit, perfusion cultivation for FW-Cs and BW-Pt could only be performed for one week.

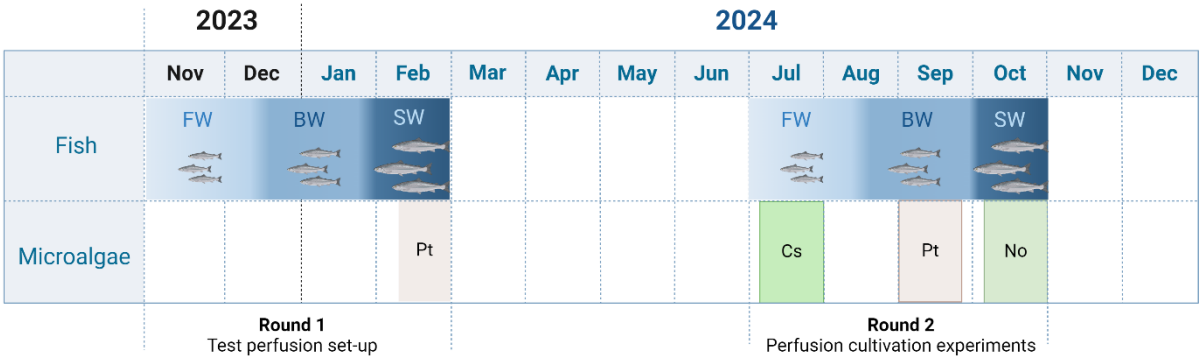

Fig. 4. Overview of the timing of the coupled fish and microalgae production. The first fish production round was used to set up and test the design of the integrated unit. The second round was used for perfusion cultivation experiments with three different microalgal species during the three salinity phases. FW=freshwater, BW=brackish water, SW=saltwater, Pt=Phaeodactylum tricornutum, Cs=Chlorella sorokiniana, No=Nannochloropsis oculata.

2.2.3 Sampling during cultivation experiments

For all four cultivation experiments, daily samples were taken from the culture for optical density (OD) and dry weight (DW) measurement to follow microalgal growth. Nitrate-N and phosphate-P concentrations were measured from the supernatant of the culture broth during batch cultivation, and from RASw-IN and the permeate during perfusion cultivation. During the cultivation experiment of Round 2 (FW-Cs, BW-Pt, SW-No) additional samples were taken to identify the impact of RAS cultivation on the microalgal biomass and to identify the

micronutrient concentration in the RAS water. These samples included bacterial counts, bacterial community composition (microbiome), dissolved organic carbon (DOC) and full elemental analysis (elements). All additional samples were stored in the fridge or freezer and will be analyzed in the coming months. An overview of the different samples taken is shown in Table 1.

Table 1. Overview of samples taken during the perfusion cultivation experiments. Samples in black were analyzed right away and samples in grey were stored and will be analyzed in coming months. FW=freshwater, BW=brackish water, SW=saltwater, Pt=Phaeodactylum tricornutum, Cs=Chlorella sorokiniana, No=Nannochloropsis oculata.

	RAS system (2x a week)	**RASw-IN (daily)**	**Culture (daily)**	**Permeate (daily)**
SW-Pt		• NO3-N • PO4-P	• Optical density (OD)	• NO3-N • PO4-P
FW-Cs	• Bacterial counts • Microbiome	• NO3-N • PO4-P • Bacterial counts • Elements • DOC	• Optical density (OD) • Dry weight (DW) • Quantum yield (QY) • Bacterial counts • Microbiome • Biomass - fatty acids - proteins - elements	• NO3-N • PO4-P • Bacterial counts • Elements • DOC
BW-Pt				
SW-No				

2.3. Analytical analysis

2.3.1 Microalgal biomass determination

Optical densities were measured in duplicate on a spectrophotometer (V-1200,VWR®) at 750 nm, diluted to give an attenuation below 0.2. Samples for dry weight determination (triplicates) were filtered on glass microfiber filters (GF/F, 47 mm, pore size 0.7 µm, Whatman International Ltd, prewashed, dried [>24 h at 95 °C] and weighed) washed three times with Milli-Q water (FW-Cs) or ammonium formate (0.25 mM for BW and 0.5 mM for SW cultures), dried in an oven at 95 C° for 24 hours and weighed (microbalance MT5, Mettler Toledo, Switzerland) after cooling off for two hours in a desiccator. Maximum quantum yield (QY) was

measured on the same sample used for OD measurements with AquaPen (AquaPen-C, AP-C 100, Photon System Instruments, Brno, Czech Republic) after an initial 2-min dark incubation.

2.3.2 Nutrient analysis

Nitrate-N (NO_3-N) and phosphate-P (PO_4^3-P) concentrations were determined in duplicates by colorimetric test kits with a compact photometer (PF-12Plus, Macherey-Nagel). Nitrate-N was measured with the VISOCOLOR ECO Nitrate kit (Macherey-Nagel), and phosphate-P was measured with the VISOCOLOR ECO Phosphate kit (Macherey-Nagel). Analyses were performed according to the kits' manual, and samples were diluted when needed, to keep concentrations within the measuring range (1 – 60 mg L^{-1} NO_{3-} and 0.6 - 15.0 mg L^{-1} PO_4^3).

2.3.3. Other analyses

Water samples for element analysis were collected in an acid-washed 500 mL plastic bottle. Samples were acidified with 1 mL nitric acid and stored in the fridge until analysis by ICP-OES. For element analysis of the biomass, microalgal biomass was collected by centrifugation, washed 2x with MQ (FW-Cs) or 1x with ammonium formate (0.25 mM, BW-Pt, SW-No) and the pellet was frozen until analysis by ICP-OES. For fatty acid analysis, biomass samples were taken by centrifugation and stored in the freezer until analysis by GC. For protein analysis, biomass was collected by centrifugation and the pellet stored frozen until analysis. For bacterial counts, volumes of 1 mL were transferred into 2 mL cryotubes, fixated with glutaraldehyde (0.5 % final conc.) at 4 ◦C for a minimum of 30 min, flash frozen in liquid nitrogen and stored at −80 ◦C until analysis. For microbiome analysis, volumes of 50 mL (culture) or 1 L (RAS water from fish tank) were stored at -20 C. For DOC analysis, 50 mL water samples were filtered (0.2 µm) and then stored at -20 C.

2.4 Calculations

Daily biomass productivity (P_B, g L^{-1} d^{-1}) was calculated from the microalgal DW (g L^{-1}) and the harvest/growth rate (H_R, d^{-1}, Eq. [1]). Daily nutrient supply (g d^{-1}) was calculated from the NO_3-N and PO_4-P concentration (N_C, P_C) of RASw-IN and the flowrate (FR) of RASw-IN (Eq. [2]). Daily nutrient removal (g d^{-1}) was calculated from the difference in nutrient concentration of RASw-IN and permeate, and the flowrate (FR) of RASw-IN (Eq. [3]). Daily nutrient removal (%) was calculated from the daily nutrient supply (g d^{-1}) and daily nutrient uptake (g d^{-1}, Eq. [4]). Calculations for nutrient uptake per biomass was calculated as shown in Eq. [5].

$$P_B = DW * H_R \quad [1]$$

$$N_{supply,\ 24h} = N_{C,RASw\text{-}IN} * FR_{RASw\text{-}IN} \quad [2]$$

$$P_{supply,\ 24h} = P_{C,RASw\text{-}IN} * FR_{RASw\text{-}IN}$$

$$N_{remove,\ 24h} = FR_{RASw\text{-}IN} * (N_{C,RASw\text{-}IN} - N_{C,permeate}) \quad [3]$$

$$P_{remove,\ 24h} = FR_{RASw\text{-}IN} * (P_{C,RASw\text{-}IN} - P_{C,permeate})$$

$$N_{remove,\ 24h}\ \% = 100/N_{,\ supply,\ 24h} * N_{remove,\ 24h} \quad [4]$$

$$P_{remove,\ 24h}\ \% = 100 / P_{,\ supply,\ 24h} * P_{remove,\ 24h}$$

$$N_{uptake,\ BM} = ((FR_{RASw\text{-}IN} / FR_{harvest}) * (N_{C,RASw\text{-}IN} - N_{C,permeate}))/DW \quad [5]$$

$$P_{uptake,\ BM} = ((FR_{RASw\text{-}IN} / FR_{harvest}) * (P_{C,RASw\text{-}IN} - P_{C,permeate}))/DW$$

3. Results

3.1 Microalgal cultivation on RAS water

In total four microalgal cultivation experiments were performed on the RAS water. The first one was a test round, growing *P. tricornutum* on seawater-RAS during round 1 of fish production (SW-Pt), where only samples for monitoring OD, DW, NO_3 and PO_4 were taken. The other three cultivation experiments were performed during Round 2 of fish production with *Chlorella sorokiniana* during freshwater phase (FW-Cs), *Phaeodactylum tricornutum* during brackish water phase (BW-Pt) and *Nannochloropsis oculata* during saltwater phase (SW-No). Here additional sampled were taken to further analyze the microalgal biomass quality and the micronutrient content in the RAS water.

3.1.1 Nitrate and phosphate concentration in RAS water

The nitrate-N concentration in the RAS water differed for the four cultivation experiments (Fig. 5 A). During round 1 (SW-Pt), nitrate-N concentration was significantly higher (82.8 mg L^{-1}) compared to round 2, where concentrations increased from fresh (26.4 mg L^{-1}) and brackish water (29.8 mg L^{-1}) to saltwater (45.2 mg L^{-1}). The strong difference in nitrate-N concentration between the two fish production rounds can be attributed to the number of fish that were present in the RAS cultivation tank, which was double as high during round 1 (515 fish) as during round 2 (200 fish). The increase in nitrate-N concentration from freshwater to seawater in round 2 is due to the increase in fish size. Black dots indicate the volume of RAS water that was flowing though the microalgal culture per day. During round 1, the flow through volume was lower with 50 L d^{-1} for SW-Pt, but was increased for the cultivation experiments in round 2 with 79 L d^{-1} for FW-Cs and 75 L d^{-1} for both, BW-Pt and SW-No.

Phosphate-P concentrations in the RAS water are shown in Fig. 5 B. The original P concentration in the RAS water was very low, during all four cultivation rounds with values between 0.7 and 1.3 mg L^{-1} (Fig. 7, lower bar). This would have resulted in high N:P ratios which would not be sufficient for microalgal cultivation (N:P > 20). Therefore, additional P was

supplemented to the ingoing RASw stream. The resulting P concentration in RASw-IN during the experiments were not at the exact intended value of N:P=5 for all cultures but were <10 for all (N:P = 9.8, 5.1, 6.5, 8.3 for SW-Pt, FW-Cs, BW-Pt and SW-No, respectively).

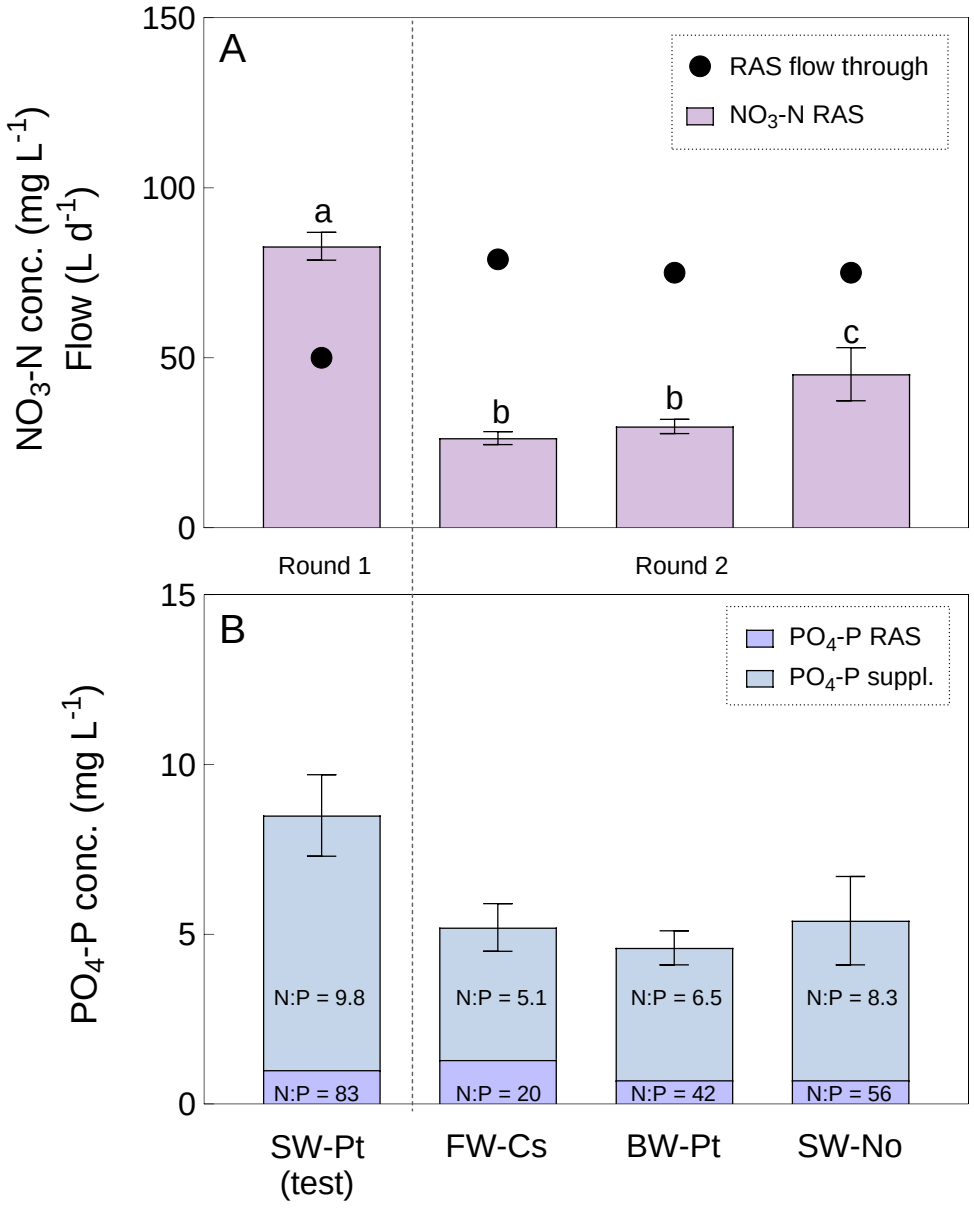

Fig. 5. Nitrate-N and phosphate-P concentration in the RAS effluent water. A: Bars show nitrate-N concentration in the RAS effluent water during the four cultivation experiments. Same letters above bars indicate no significant differences (p > 0.05). Black dots show the volume of RAS effluent water flowing through the reactor in one day. B: Phosphate-P concentrations in the RAS water during the four cultivation experiments. Lower bars shows the original Phosphate-P concentrations in the RAS effluent water and the higher bar shows the concentration after P supplementation. N:P values are given for both original RAS water and supplemented RAS effluent water. FW=freshwater, BW=brackish water, SW=saltwater, Pt=Phaeodactylum tricornutum, Cs=Chlorella sorokiniana, No=Nannochloropsis oculata. *Values for NO3-N and PO4-P suppl. are average and standard deviation for measurements from 5 (SW-Pt, FW-Cs, BW-Pt) or 10 (SW-No) days. Values for PO4-P RAS is from one day (before experiment start).*

3.1.2 Microalgal growth during cultivation

Growth curves for the whole cultivation period of the four separate microalgal experiments are shown in Fig. 6. Each cultivation started with a batch cultivation to reach the desired biomass concentration for perfusion cultivation. The microalgal biomass concentration that can be maintained in the reactor, was dependent on the nitrate-N concentration and volume of RAS effluent water provided per day. Perfusion cultivation was performed for eight days for SW-Pt, nine days for FW-Cs and BW-Pt and 12 days for SW-No. Perfusion cultivation was shorter for FW-Cs and BW-Pt compared to SW-No, because of problems with the membrane filtration unit, where cultivation had to be stopped. During perfusion cultivation an average OD of 4.3 and 4.0 was reached for FW-Cs and BW-Pt, respectively, while higher ODs were reached for SW-No (7.3) and SW-Pt (10.9), which was due to the higher NO_3-N concentrations in the RAS effluent water. The biomass concentration increased slightly for *P. tricornutum* in both SW and BW perfusion cultivation. This is most probably due to that the chosen harvesting rates were a bit lower than the actual growth rates, leading to an increase in biomass density in the reactor. The maximum quantum yield remained stable during the whole cultivation period for all cultures. A decreasing maximum quantum yield is an indicator of stress, such as nutrient depletion, and was used to confirm a stable photosynthetic activity during the cultivation experiments. Days of stable perfusion cultivation are indicated with green circles in Fig. 6, and were used for calculating microalgal productivities and nutrient removal.

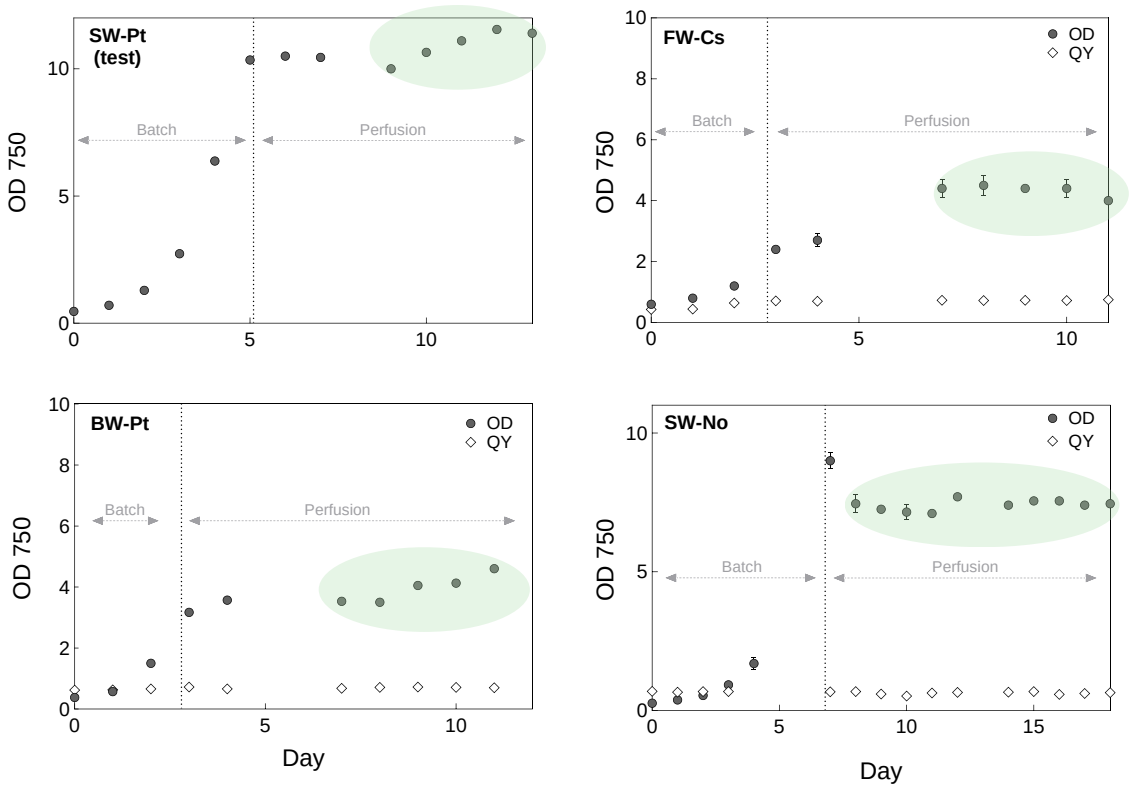

Fig. 6. Growth curves for the whole cultivation period of the four separate microalgal experiments. FW=freshwater, BW=brackish water, SW=saltwater, Pt=Phaeodactylum tricornutum, Cs=Chlorella sorokiniana, No=Nannochloropsis oculata, *OD= optical density. QY= maximum quantum yield. Green areas indicate days of stable perfusion cultivation that were used for calculating microalgal productivities and nutrient removal.*

3.2 Productivity and nutrient uptake during perfusion cultivation

Microalgal productivities and nutrient removal were calculated for each cultivation experiment from several days of stable perfusion cultivation. This was five days for SW-Pt, FW-Cs, and BW-Pt, and 10 days for SW-No (Fig. 6, green areas). The daily nutrient supply (nitrate-N and phosphate-P) provided for the microalgal culture was calculated from the concentration in the ingoing RAS effluent water and the RAS volume that was flowing through the reactor in 24 h (Fig. 7 A). Nitrate-N amounts provided per day was highest for SW-Pt (4.1 g d^{-1}) followed by SW-No (3.4 g d^{-1}), BW-Pt (2.2 g d^{-1}) and FW-Cs (2.1 g d^{-1}). This was due to the higher N concentrations in the RAS effluent water during round 1 and a general increase in N concentration from fresh to saltwater. This resulted in highest average DW concentrations for SW-Pt (3.6 g L^{-1}), followed by SW-No (2.5 g L^{-1}), BW-Pt (1.3 g L^{-1}) and FW-Cs (1.1 g L^{-1}) during

perfusion cultivation (Fig. 7 B). Growth rates were highest for *C. sorokiniana* with an average growth rate of 1.2 d^{-1} (Fig. 7 D). Growth rates for *P. tricornutum* were higher in brackish water cultivation compared to saltwater cultivation (0.8 and 0.5 d^{-1}, respectively). This could be due to that *P. tricornutum* growth better at lower salinities but could also be connected to the lower total irradiance the cells received during SW cultivation, as the biomass concentration in the reactor was much higher compared to BW cultivation. *N. oculata* had an average growth rate of 0.8 d^{-1}. The total biomass production for each culture was calculated from the DW and the growth rate and resulted in highest productivities for *N. oculata* (SW-No) with 1.9 g L^{-1} d^{-1}. *P. tricornutum* had higher biomass productivities during SW cultivation (SW-Pt) with 1.8 g L^{-1} d^{-1} compared to 1.1 g L^{-1} d^{-1} during BW cultivation (BW-Pt). *C. sorokiniana* had an average biomass production of 1.3 g L^{-1} d^{-1}.

The nutrient removal was calculated from the nutrient supply through the ingoing RAS effluent water and the amount of nutrients left in the permeate. *P. tricornutum* removed 100% of the nitrate and phosphate in both, SW and BW cultivation (Fig. 7 C). *C. sorokiniana* removed 99% of the nitrate and 90% of the phosphate, while *N. oculata* removed 80% of the nitrate and 100% of the phosphate. The lower nitrate uptake of *N. oculata* could indicate phosphate limitation. The average N:P ratio in the ingoing RAS water was 8.3. If *N. oculata* has a higher P requirement, the growth could have been limited by the P availability, and hence not all of the nitrate could be utilized. The nitrate-N uptake per biomass is shown in figure Fig. 7E. The amount of N and P that was taken up per gram microalgal biomass were in the range that is generally reported for microalga (1% for N and 0.1% for P). However, the exact values differed between the species. N uptake and was highest for *P. tricornutum* with 0.11 g g^{-1} d^{-1} and was the same for both, saltwater and brackish water cultivation. The P uptake, however, was higher during cultivation in brackish water with 0.017 g g^{-1} d^{-1} than saltwater cultivation (0.012 g g^{-1} d^{-1}). This was due to the lower N:P ratio (higher P concentration) in the RAS water during brackish water cultivation (N:P brackish = 6.5, N:P salt = 9.8). *P. tricornutum* is known to perform a process called luxury uptake, where microalgae take up more phosphorous than necessary for immediate growth and accumulate it within their cells as polyphosphate. *C. sorokiniana* and *N. oculata* had an N uptake of 0.08 and 0.07 g g^{-1} d^{-1} and a P uptake of 0.014 g g^{-1} d^{-1}, and 0.011 g g^{-1} d^{-1}.

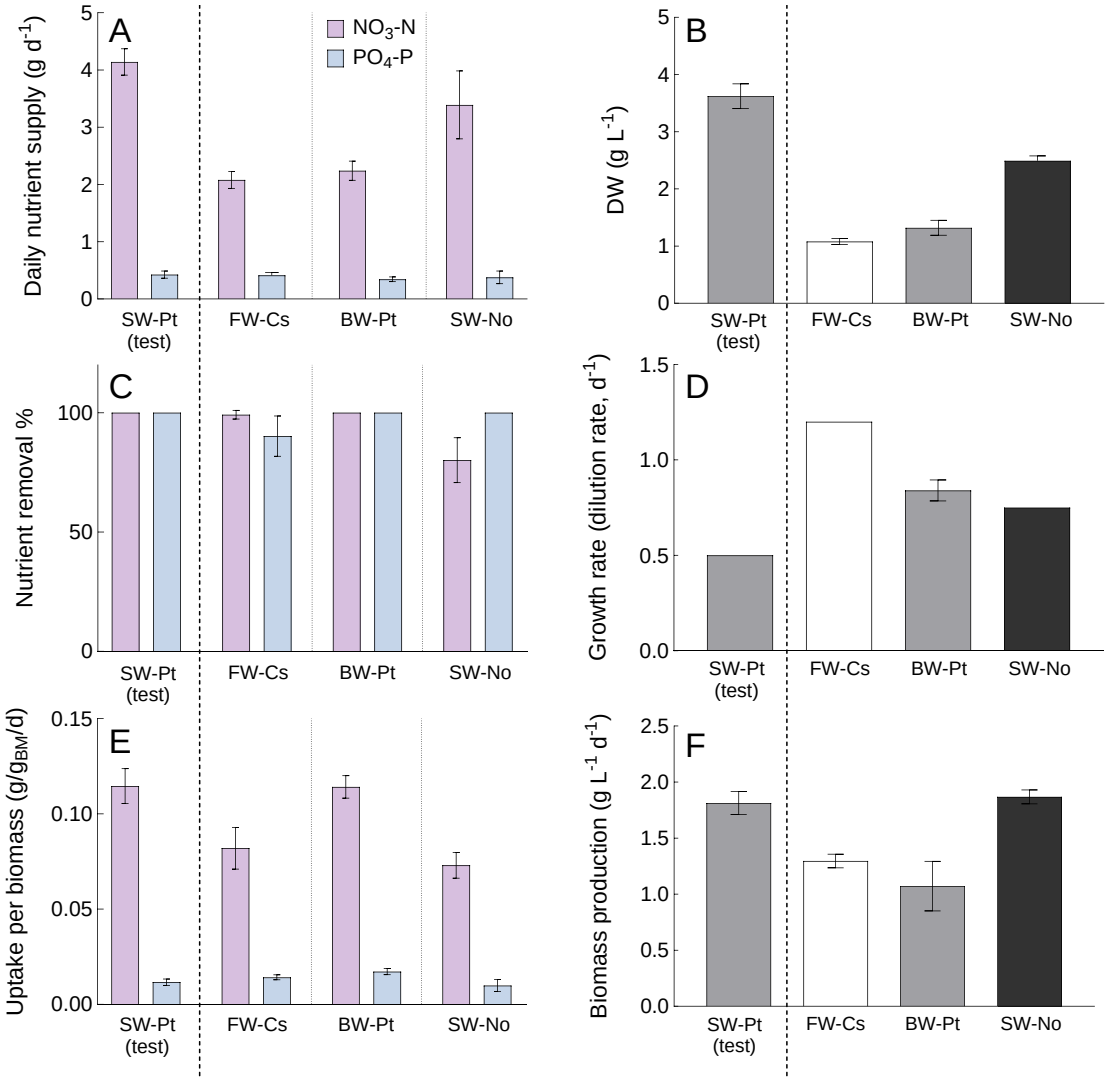

Fig. 7. Nutrients (nitrate-N and phosphate-P) and biomass parameters during perfusion cultivation of the four separate microalgal experiments. A: daily supply of nitrate-N and phosphate-P. B: Biomass dry weight (DW). C: percentage of nitrate-N and phosphate-P removal. D: growth rates. E: nitrate-N and phosphate-P uptake per biomass. F: Daily biomass production. Values are average and standard deviation for measurements from 5 (SW-Pt, FW-Cs, BW-Pt) or 10 (SW-No) days. FW=freshwater, BW=brackish water, SW=saltwater, Pt=Phaeodactylum tricornutum, Cs=Chlorella sorokiniana, No=Nannochloropsis oculata.

4. Conclusion

During perfusion cultivation, high microalgal productivities and high removal of nitrate and phosphate from the RAS effluent water could be achieved during the whole cultivation period (fresh, brackish and seawater). Removal capacities differed between the microalgal species. *P. tricornutum* removed 100% of nitrate-N and phosphate-P from the RAS water while *C. sorokiniana* removed 99% of the nitrate and 90% of the phosphate, and *N. oculata* removed 80% of the nitrate and 100% of the phosphate. These removal rates can be related to the availability of other nutrients such as phosphate. Hence, providing nutrients in a sufficient ratio is crucial to achieve maximized nutrient removal.

The microalgal biomass productivities were dependent on the nitrate concentration in the RAS effluent water, but also the volume flowing through the system and the growth rate of the microalgae. By increasing the flow through of RAS through the system, and optimizing other parameters such as salinities, irradiance and temperatures can help to optimize and further increase productivities. Coupling smart online monitoring technologies based on optical spectroscopy such as multispectral turbidity, nutrients and biomass content to the RAS-microalgal unit, will allow a better steering for optimal conditions like retention times and biomass concentration. Moreover, information on the quality of the treated water and of the produced microalgal biomass from the currently ongoing analysis will allow us to show the suitability of this process both regarding the re-use of the water for the fish production, thus lowering the use of water in the facility, and regarding the use of the microalgal biomass towards food production, as studied in the subsequent work packages.

5. References

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