DTU Aqua
National Institute of Aquatic Resources

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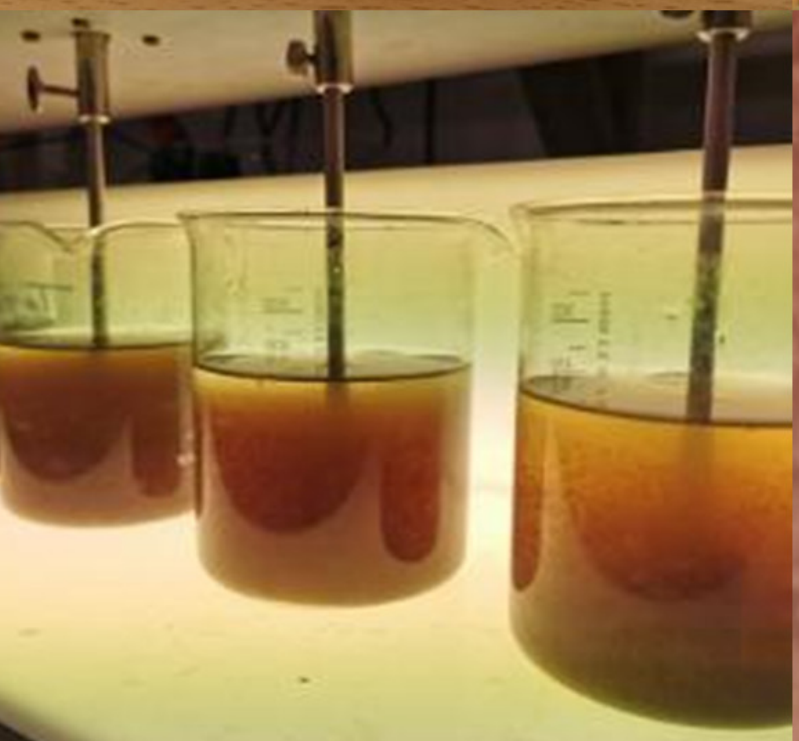
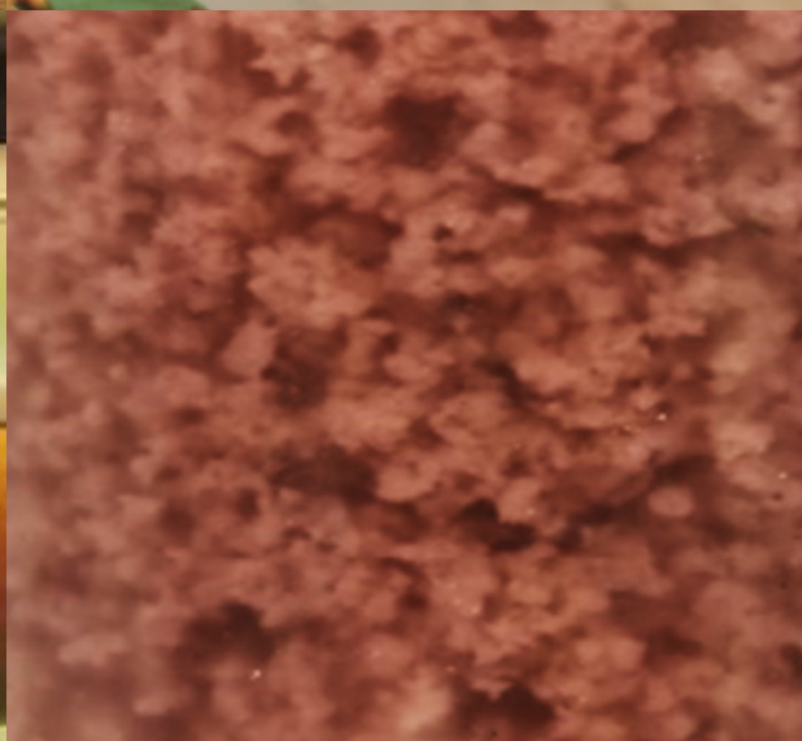
DTU Aqua Report no. 479-2025





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Colophon

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Authors:	Carlos O. Letelier-Gordo[1], Ching Seow[1], Jaime Pons[1], and Laura Hernandez[2] [1] DTU Aqua [2] Alumichem
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Preface

The purpose of the project is to develop technical and scientific knowledge within end-of-pipe treatment for the implementation of innovative cost-efficient technologies for the removal of nitrogen (N), phosphorous (P) and organic matter (OM) in Model farms type 1, 3 and FREA. The technologies have a reduced resource usage, easy operability and capable of working on demand, adjusting the end-of-pipe operational costs to the farm production level, while decoupling economic growth from environmental burden. By this strengthening the sector competitiveness, helping to drive food security in Denmark and the EU while providing significant economic benefits and job security (SMEs in rural/coastal regions). The WP1 (N removal) and WP2 (P and OM removal) are focused on the development of technical and scientific knowledge starting at lab scale trials to decipher the optimal resource/operational parameters for further application and demonstration under industrial conditions in fresh and marine water (Model type 3 and Freia). WP3 oversees the valorisation of the fish organic waste as a residual resource and its incorporation in the value chains (e.g., fertilizer composting and P recovery), while WP4 is focused on dissemination and communication of the project results. The expected effects of the proposed technology are to allow farms to at least increase 50% the farm production capacity based on N quota. A dynamic WP4 plan was developed at the beginning of the project with the objective of communicating and disseminating the goals, challenges and relevant results from the project to the stakeholders, policy makers and public through social media, webinars, workshops, conferences and specialized magazines.

The project partners belong to different value chains in the aquaculture sector. Alumichem was leader of WP2 P removal technologies, Alpha Aqua was in charge of system design and holistic approaches to end-of-pipe technology, Dansk Akvakultur engaged in discussions on fish manure valorisation and legislations, Danforel allowed the evaluation of the technologies in Christiansminde and Aqua Circle helped organizing the final project workshop. Further details of each partner role in the project can be found in acknowledgments section.

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Hirtshals, December 2023

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Content

Summary

The project aims to reduce the emission of nitrogen (N), phosphorus (P) and organic matter (OM) through the development of two innovative and practical final cleaning solutions: I) Flocculating bacteria; a technology based on the formation of cooperative bacterial colonies that significantly increases the overall cleaning of dissolved substances. II) A new cost-effective technology where organic/degradable flocculants and lignocellulosic components are used to effectively precipitate P and OM.

The project results from AP1 presents an innovative technology based on flocculant bacteria allowing more than 90% N removal with the potential to be applied in most of the Danish farms using RAS technology and provides the Danish equipment industry with new technologies to add into their portfolio. An evaluation of different removal reactors at different TRL levels using flocculant bacteria was developed. The reactors were activated sludge (AS) TRL8, fed batch reactor (FBR) TRL5 and upflow anoxic sludge blanket (UASB) TRL4. All reactors managed to remove >90% N treating at different capacities (82-2400 gN/m3 reactor) and configurations giving alternatives to be applicable at different Danish farms using flocculant bacteria technology.

Nitrous oxide is a greenhouse gas (GHG) equivalent to 300 times CO_2 and it is a step in the bacterial process of removing N. The main process for removing N from water applied in Danish farms as wetlands, woodchips and flocculant bacteria technologies (this project). We determined that the production is related to the carbon to nitrate ratio (C:N). In systems where passive carbon supplementation is done (wetlands and woodchips) the process is relatively uncontrolled while in flocculant reactors carbon is administered, and nitrous oxide production can be reduced significantly at a C:N ratio of 6 or higher. In the tested technology of this project, 5.6% of nitrous oxide (greenhouse gas) was produced per amount of nitrogen treated. Nitrous oxide is not regulated today but considering the European green deal, this emission needs eventually to be accounted for.

The project results from AP2 present a profound evaluation of different coagulants and flocculants for the removal of phosphorous and organic matter, achieving removal rates >90% for both parameters. Further-more, a new technology that utilizes lignocellulosic and cellulosic materials in combination or not with coagulants/flocculants allows the production of a fish manure cake of 25-35% dry matter, reducing transportation weight by 80% as compared to the actual situation (fish sludge), as well as reductions of 80% organic matter, 85% P and 78% N from the treated sludge. The technology is applicable at organic farms, Model Trout Farms 2 & 3 and FREA.

The fish manure cake has a total composition of 31% dry matter, 0.16% N and 0.2% P and significant reduction in heavy metals (10-fold below the Danish legislation), making it suitable for composting and low intensity fertilizer. Giving the fish farmer an alternative for reducing transportation cost while valorising the fish manure. The technology can potentially be tailored, and additional elements can be added to in-crease the fertilizer properties. We used struvite (P recovery form) and we managed to significantly increase N and P, reaching 34% dry matter, 5% nitrogen and 2% phosphorus. To prove the concept, we grew

mushrooms in freshwater and saltwater fish manure cake demonstrating the potential of the fish manure cake to be used as compost or fertilizer material and treatment method for saline fish manure.

The project results have been disseminated and communicated through different medias and stakeholders:

- Conferences
- Stake holder meetings
- Magazine articles
- Social media
- Education
- Workshops
- Peer review scientific publications.

Danish summary

Projektet havde til formål at reducere udledningen af kvælstof (N), fosfor (P) og organisk materiale (OM) fra fiskeopdræt i Modeldambrug (type 1 og 3) og fuldt recirkulerede anlæg (FREA) gennem udvikling af to innovative og praktiske slutrensningsløsninger: I) Bakteriel flokkulering, en teknologi baseret på kooperativ dannelse af bakteriekolonier der bidrager til at øge den samlede rensning af opløste stoffer betydeligt, og II) Anvendelsen af flokkuleringsagenter, en ny omkostningseffektiv teknologi, hvor organiske/nedbrydelige flokkuleringsmidler og lignocellulose komponenter anvendes til en omkostningseffektiv udfældning af P og OM.

Projektets resultater fra AP1 præsenterer en innovativ teknologi baseret på flokkulerende bakterier, der muliggør mere end 90% N-fjernelse med potentiale for anvendelse i de fleste danske recirkulerede fiskeopdræt, og som bidrager med nye teknologier til de danske udstyrsleverandørers porteføljе. Reaktorer baseret på flokkulerende bakterier blev evalueret på flere TRL-niveauer under anvendelsen af aktiveret slam (AS, TRL8), fodret batchreaktor (FBR, TRL5), og opstrøms anoksisk slamtæppe (UASB, TRL4). Alle reaktorer formåede at fjerne >90% total N om end med forskellige kapaciteter (82-2400 gN/m3 reaktor) og konfigurationer, hvilket giver forskellige alternativer for implementeringen af flokkuleringsteknologi på danske opdrætsanlæg.

Lattergas er en drivhusgas (GHG), der er 300 kraftigere end CO2, og udgør et trin i den bakterielle omsætning af N. Slutrensning af N på danske opdrætsanlæg er typisk gennem vådområder eller træflisfiltre, eller flokkulering med bakterieteknologier (dette projekt). Vi fastslog, at produktionen af lattergas er relateret til forholdet mellem kulstof og nitrat (C:N). I systemer hvor passivt kulstoftilskud udføres (vådområder og træflis) er processen relativt ukontrolleret, mens der i flokkuleringsreaktorer kulstoftilførsel er kontrolleret, kan lattergasproduktionen reduceres betydeligt, såfremt der opretholdes et C:N-forhold på 6 eller højere. Med den afprøvede teknologi i dette projekt blev der produceret 5,6% lattergas (drivhusgas) pr. mængde behandlet kvælstof. Lattergas er ikke reguleret i dag, men i betragtning af den europæiske grønne pagt skal der i sidste ende tages højde for denne emission.

Projektets resultater fra AP2 præsenterer en dybdegående evaluering af forskellige koagulanter og flokkuleringsmidler til fjernelse af fosfor og organisk materiale, hvor fjernelsesrater på >90% blev opnået for både P og OM. Ydermere viser vi, at anvendelsen af teknologier med lignocellulose- og celluloseholdige materialer, i kombination med koagulerings- og flokkuleringsmidler, at der kan produceres en pressekage af fiskegødning med 25-35 % tørstofindhold. Dette reducerer transportvægten med 80 % i forhold til ubehandlet (vådt) fiskeslam, og en reduktion på 80 % OM, 85 % P og 78 % N fra det behandlede slam. Teknologien er desuden anvendelig på økologiske opdræt, modeldambrug type 2 & 3, og FREA-anlæg.

Pressekagen har en sammensætning på 31 % tørstof, 0,16 % N og 0,2 % P og en betydelig reduktion af tungmetaller (10 gange lavere end tilladt under dansk lovgivning), hvilket gør den velegnet til kompostering eller lavintensiv gødning. At give fiskeopdrætteren et al-

ternativ til at reducere transportomkostningerne, samtidig med at fiskegødningen valoriseres. Teknologien kan potentielt skræddersys, f.eks. ved tilføjelse af yderligere næringssalte eller mineraler for at øge gødningens egenskaber. Vi anvendte struvit (P-genvindingsform), og det lykkedes os at øge N og P betydeligt og nå op på 34 % tørstof, 5 % nitrogen og 2 % fosfor. For at validere konceptet dyrkede vi svampe i pressekage produceret fra ferskvands- og saltvandsopdræt, og demonstrerede at pressekagen har potentiale til at blive brugt som kompost eller gødningsmateriale, og at behandlingsmetoden også kan anvendes i slam fra saltvandsopdræt.

1. Nitrogen removal (denitrification)

Heterotrophic denitrification is the main NO_3^- removal technology applied in RAS and comprise of a sequential process with four enzymatic steps, including reduction of nitrate to nitrite (NO_2), nitric oxide (NO), nitrous oxide (N_2O), and finally to nitrogen gas (N_2) (Henze et al., 1997). Denitrifying bacteria use a wide spectrum of organic carbon sources that can be obtained either commercially (external carbon sources) or from RAS effluent (internal carbon sources). Methanol and ethanol are the common choices of external carbon source for RAS (van Rijn, 2013). However, being reactive flammable alcohols, they require special standards for transport, storage, packaging, handling, and disposal (European Parliament and the Council of the European Union, 2008) adding additional capital costs(Cherchi et al., 2009). Alternative carbon sources such as volatile fatty acids (VFA) (e.g. acetate, propionate, butyrate), obtained either commercially or generated from fish waste (Aboutboul et al., 1995; Letelier-Gordo et al., 2020b; Suhr et al., 2015a) are thus an interesting alternative. Furthermore, the utilization of fish waste for carbon sources can reduce the cost of external carbon source while creating a residual resource or value from waste.

Media-laden reactors are the most popular technology in aquaculture at brackish or marine water conditions (Balderston and Sieburth, 1976; Grguric et al., 2000; Gutierrez-Wing et al., 2012; Honda et al., 1993). However, channelling, clogging and increased pressure drops due to organic matter accumulation, are common when media-laden reactors are used for the denitrification process (Balderston and Sieburth, 1976; Müller-Belecke et al., 2013; Sauthier et al., 1998). This might reduce the effective denitrification capacity of the reactor, requiring frequent backwashing to sustain continuous operation. Organic matter accumulation is especially problematic in marine systems, since the anaerobic conditions will promote the production of toxic H_2S (Letelier-Gordo et al., 2020a; Rojas-Tirado et al., 2021). Therefore, marine land-based facilities require alternative solutions for media-laden denitrification reactors with simple construction and operation, high denitrification rates, and a low footprint.

The use of bacterial flocs or flocculent bacteria (e.g. activated sludge) is globally the most common treatment technology for biological nitrogen (N) and organic matter removal in municipal and industrial wastewater treatment (Henze et al., 1997a; Tchobanoglous et al., 2002). Unlike media-laden reactors, flocculent bacteria live suspended inside the reactors without the need for plastic carrier elements. Depending on the type of floc developed, high denitrification rates can be achieved (12-14 kg NO_3--N/m^3 of reactor per day) (Klapwijk et al., 1981; Letelier-Gordo and Herreros, 2019).

Nitrite an intermediate compound during denitrification process can accumulate at high concentrations in the body fluids of aquatic organism (Banerjee et al., 2023). As such, fish has a high risk of nitrite intoxication which can lead to organ damage and decreased immunological functions (Banerjee et al., 2023). NO is also a toxin, and nitrous oxide is a potent greenhouse gas and a key ozone- depleting agent (Ni et al., 2011). As such, it is crucial to under-stand the effect of C/N ratios and HRT in conjunction with the selected system design, to ensure that complete denitrification is achieved, avoiding the production of intermediate compounds which are toxic to marine organisms and create greenhouse gases.

1.1 Carbon to nitrogen ratio

The C/N ratio in denitrification reactors are typically maintained through the addition of exogenous carbon sources such as methanol and acetate, which are easily de-graded by denitrifiers (Chiu and Chung, 2003; Constantin and Fick, 1997; Gao et al., 2020; Lee and Welander, 1996; Letelier-Gordo et al., 2020b; Narkis et al., 1979; Suhr et al., 2015a, 2015b, 2013; Xu, 1996). This can be complemented by endogenous carbon, i.e. organic carbon from fish faeces. This in turn reduces production costs and the risk associated with transporting and storing these hazardous chemicals (Letelier-Gordo et al., 2020b). To make endogenous carbon accessible to denitrifiers, organic waste must be hydrolysed and fermented to produce volatile fatty acids (Suhr et al., 2015b, 2015a). It is generally agreed that a Chemical Oxygen Demand (COD) /N ratio of 3-6 would allow for complete denitrification (van Rijn et al., 2006, 1995). The C:N values depend on the incoming oxygen concentration of the system as denitrifying bacteria is facultative and they will firstly use oxygen for energy before denitrification occurs. Therefore, it is mandatory to remove oxygen from the water to promote denitrification process and account the carbon required for this in the C:N ratio.

1.2 Hydraulic retention time (HRT)

HRT impacts the performance of the denitrification reactor as it influences the amount of contact time de-nitrifiers have, to convert nitrate to nitrogen gas (Henze et al., 1997a). The optimal HRT is dependent on other factors which affect denitrification efficiency such as the C/N ratio, denitrifying reactor biome and bacterial biomass (e.g. flocs, granules). When C/N ratio is more than 6, short HRT of 1.9 hours had been achieved with granules to remove 14.9 kg NO_3-N/m^3 reactor volume per day (Klapwijk et al., 1981; Letelier-Gordo and Herreros, 2019). As such, the shorter the HRT, the higher the treatment efficiency which in turn allows for a greater removal of NO3-N.

1.3 System design

Bacteria can form flocs in activated sludge systems via a selection process i.e. by selecting heavier aggregates that can settle in the reactor and washing out the rest (Letelier-Gordo and Herreros, 2019). Flocculent bacteria are commonly used for denitrification in wastewater treatment (Tchobanoglous et al., 2002). A key advantage is that flocculent bacteria do not need a carrier element and live suspended in the denitrification reactor (Letelier-Gordo et al., 2020b; Letelier-Gordo and Herreros, 2019; Suhr et al., 2015a). High denitrification rates have been proven from the use of these flocs, though nitrogen removal rates depend on the type of flocs, bacterial biome and count.

1.3.1 Continuous stirred reactor (CSTR) and activated sludge systems

There are several considerations when designing a denitrification reactor. Firstly, if a continuous stirred-tank reactor (CSTR) is employed, a settling tank is required. This is to increase solid retention time (SRT) by retaining the bacteria within the denitrification tanks. Without a settling tank, a lower HRT than the SRT would result in the bacteria/biomass being washed out of the denitrification process tanks. However, this design requires space and involves the construction of a settling tank increasing construction and energy costs due to recycling of the bacterial biomass back to the process tank. The core of the activated sludge system is the formation of a flocculent bacteria with high settling capacities.

This characteristic of the bacteria is exploited using a settler and a pump, having respectively the function of collecting and recirculating the bacterial mass back into the reactor, hence, increasing the time the bacteria spend inside the reactor or the solid retention time (SRT). Through this action, the bacteria mass becomes independent from main flow of the reactor or the hydraulic retention time (HRT), complying with the same purpose as using inert carriers in media-laden reactors.

1.3.2 Fed batch reactor (FBR)

A FBR operates in a step wise cycle in the following order: 1) filling the FBR with effluent water to be treated, 2) mixing at a set HRT to allow for denitrification to occur, 3) settling of denitrifying bacteria for subsequent batch treatment and 4) discharge of treated effluent water from the top of the reactor. It was demonstrated that FBR had the potential to be used as a denitrifying EOP technology in RAS operations (Letelier-Gordo et al., 2020b). It was concluded that FBR was a suitable due to the easy setup and maintenance, small footprint, reliable nitrogen removal and low operational costs.

In municipal wastewater treatment, FBR has been broadly studied (Wang et al., 2010), but the applicability of FBR for N removal in marine RAS, or aquaculture in general, is not currently known or applied.

1.3.3 Upflow anoxic sludge blanket (UASB)

Upflow Anaerobic/Anoxic Sludge Bed (UASB) is a type of reactor type utilizing flocculant ort granular sludge. The reactor does not require an inert carrier (plastic), can achieve very efficient mixing, does not present clogging problems, and it is widely used in high-rate anaerobic wastewater treatment systems (Henze et al., 2008). In a UASB reactor, water enters at the bottom creating an upward plug-flow that maintains the flocs in suspension. The key indicators of granular sludge formation and correct system performance are: 1) biomass composition, 2) the Sludge Volume Index (SVI), and 3) the upflow velocity (Vu). The SVI is an indicator of the settling capacity of the granular sludge (APHA, 2012). The upflow velocity is the velocity at which the water rises from the bottom to the upper part of the UASB. These types of reactors are mostly used for biogas production while their use in denitrification systems is quite limited. They have a big potential in aquaculture applications due to their high volumetric N removal capacity (14 kgN/m^3 reactor day) (Klapwijk et al., 1981; Letelier-Gordo and Herreros, 2019). The drawback is the formation of the flocculant bacteria and the capacity to maintain the bacteria inside the reactor due to N_2 gas formation in the flocs.

1.4 Endogenous carbon sources

Organic matter and nitrate are two major effluent waste products in a RAS, and cost-efficient capture and handling of these waste products are major challenges that commercial inland aquaculture must address to become more environmentally sustainable. Furthermore, the use of external carbon sources and energy should be limited to effectively accommodate these challenges.

Endogenous carbon sources for denitrification in RAS complies uses the organic waste produced by the fish (faecal matter) as an internal electron donor for nitrate removal (Elefsiniotis et al., 2004; Hamlin et al., 2008; Suhr et al., 2014, 2013). In this way, organic

matter is used to remove nitrate without or to some extent the need for external carbon as methanol or acetate. In addition, the transport of waste is reduced, and most importantly the waste is treated at the site in the end of pipe process chain, rather than being displaced to another environment (end-of-pipe concept) (Glavič and Lukman, 2007).

Studies exploring the potential of using fish organic waste for denitrification in RAS have been carried out for the last 20 years and have included a fermentation step to enhance the production of readily available carbon sources as volatile fatty acids (VFA) specifically; acetate, propionate, butyrate, valerate and formic acid (Letelier-Gordo et al., 2015).

The composition of the readily available carbon sources (RACS) has been shown to affect denitrification rates, sludge production, and denitrification yields (Henze et al., 1997a; Van Rijn et al., 1996). Different authors have demonstrated that denitrification rates using acetic acid or a mixture of VFAs as electron donors may reach twice the turnover compared to using methanol (Fass et al., 1994; Lee and Welander, 1996; Xu, 1996), which is often applied as an external carbon source in aquaculture. In similar studies it has been shown that propionate reduces denitrification rates by half compared to acetate, butyrate and valerate (Elefsiniotis and Wareham, 2007). Moreover, different C:N ratios (Xu, 1996) and bacterial yields (Constantin and Fick, 1997) have been reported when using different organic carbon sources.

As opposed to many other types of wastewaters, faecal waste in RAS is produced in a continuous and predictable manner, both in terms of quantity and quality. The particulate waste is mainly composed of the undigested fractions of commercial feed deriving from a predefined amount of feed (i.e., input) fed into the system each day. This means that aquaculture faecal waste has good potential as a constant residual resource for biological waste treatment. Furthermore, the proximate composition of commercial fish feed is generally well described, and the digestibility of most commercial ingredients is well established, at least in rainbow trout (Oncorhynchus mykiss). It is therefore possible to couple feeding of rainbow trout with the quantity and nutrient composition of the waste produced in the system (Dalsgaard and Pedersen, 2011). The production and application of specific types of organic acids obtained from fish faecal waste via incomplete anaerobic digestion will consequently reduce operational costs while creating a value from waste.

1.5 Research aims for denitrification

The aim objectives of this part of the study were to:

- Develop flocculent denitrifying bacteria,
- Investigate the effect of different C/N ratios on denitrification rates and intermediate products in freshwater and saltwater conditions
- Design and evaluate a FBR using the flocculent denitrifying bacteria at different HRT,
- Evaluate the denitrification performance of a commercial saline activated sludge RAS.

1.6 Materials and methods

1.6.1 Formation of denitrifying flocculent bacteria

Denitrifying flocculent bacteria was prepared by conditioning organic waste from a freshwater and saltwater rainbow trout (*Oncorhynchus mykiss*) RAS. 5L of fish organic waste was placed in 10 L Pyrex® media bottles (Sigma-Aldrich, Denmark) at a concentration of 10 g/L COD and the remaining volume was filled with freshwater. The 10 L bottles were operated in a step fed-batch manner with one cycle per day. The mixing phase consisted of 23.5 h at 200 rpm using magnetic stirrers (IKA C-Mag MS 7, Germany). The settling period consisted of 30 minutes in which after 40% of the supernatant was discharged and posteriorly filled, corresponding to one cycle. The filling medium consisted in freshwater with the addition of sodium acetate (C2H3NaO2, >99%, GPR RECTAPUR@, VWR, USA) and sodium nitrate (NaNO3, >99%, Acros Organics, USA) to obtain a final concentration of 50 NO3-N mg/L and a final COD:NO3-N of 6.

1.6.2 C/N ratio batch trial

Saltwater trials

Denitrification was examined in activated sludge with different C:N ratios: C:N = 0.5, C:N= 1.5, C:N = 2.7 and C:N = 4. Each C:N ratio was set up as 3 biological replicates containing activated sludge (biomass ranging from 5.2 +- 0.7 g xCOD/L). C:N ratios were reached by adding sodium acetate as carbon source and potassium nitrate. Measurements were conducted on soluble N_2O, H_2S, NO_2^-, NO_3^-, pH and ORP over 24 hours in 2L bluecap bottles under magnetic stirring. Conditions were set to mimic those in the RAS commercial facility with a temperature between 14.6 – 15.7 °C and salinity between 33-35 ppt.

Freshwater trials

Denitrification performance was assessed with three different C:N ratios: 3.6 ± 0.0, 4.7 ± 0.3, 7.1 ± 0.1, using twelve 2L Blue Cap glass bottles. Each C:N ratio was conducted in triplicates, with sodium acetate added to reach the abovementioned C/N ratio while maintaining NO_3^--N concentrations at approximately 50 mg/L. A control was also conducted in triplicate where only acetate was added to achieve a concentration of 364 mg/L SCOD i.e. no NO_3^--N in the control. The experiment was conducted at a temperature of 15.1 ℃ and the bottles were mixed with magnetic stirrers at 200 rpm (IKA, Germany). The average particulate COD (xCOD) concentration in each bottle was 1.73 ± 0.3 g/L.

Approaches and calculations

Specific denitrification rates (SDNR) were calculated using

$$SDNR = \frac{[NO_3^- - N\ start]\left(\frac{mg}{L}\right) - [NO_3^- - N\ steady\ state]\left(\frac{mg}{L}\right)}{time\ (h)\ x\ [Activated\ sludge\ biomass]\ \left(\frac{g}{L}\right)}$$ (Equation 1)

Specific NO_2^- production rates were calculated using (Equation 2)

$$\boldsymbol{SNO_2^-PR = \frac{[NO_2^-\ steady\ state]\left(\frac{mg}{L}\right) - [N_2O\ start]\left(\frac{mg}{L}\right)}{time\ (h)\ x\ [Activated\ sludge\ biomass]\ \left(\frac{g}{L}\right)}}$$

Specific N_2O production rates were calculated using (Equation 3)

$$SN_2OPR = \frac{[N_2O\ max]\left(\frac{mg}{L}\right) - [N_2O\ start]\left(\frac{mg}{L}\right)}{time\ (h)\ x\ [Activated\ sludge\ biomass]\ \left(\frac{g}{L}\right)}$$

1.7 Results and discussion

1.7.1 C/N batch trial freshwater

For the first 12 hours, the reduction in nitrate (Fig. 1) strongly correlated with nitrite accumulation for all treatments (Fig. 2) (R^2 = 0.996). While the pH continued to decrease for the control reactors after 12 hours, the pH of all other reactors started to increase. This corresponded to the complete depletion of nitrate in the reactors, accumulation of nitrite and production of nitrous oxide (N_2O-N) (Fig 3).

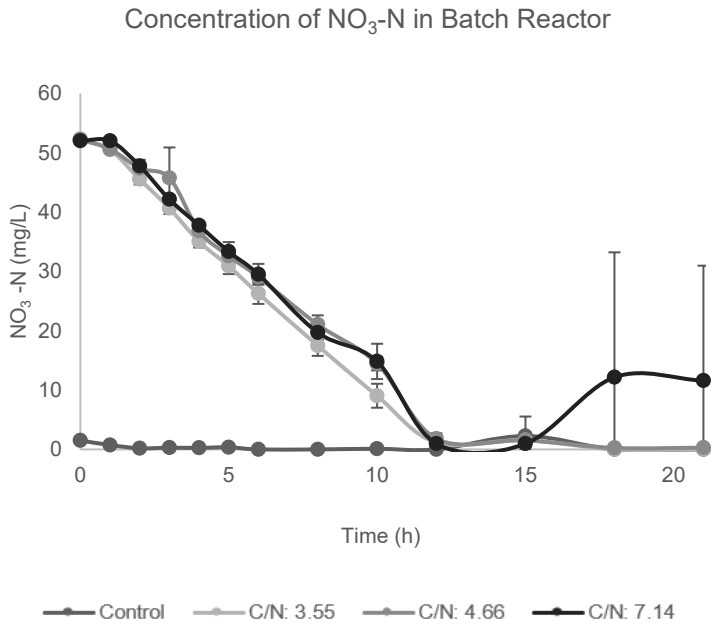

Figure 1. Nitrate concentration dynamics in batch reactor trials at different C:N ratios in freshwater.

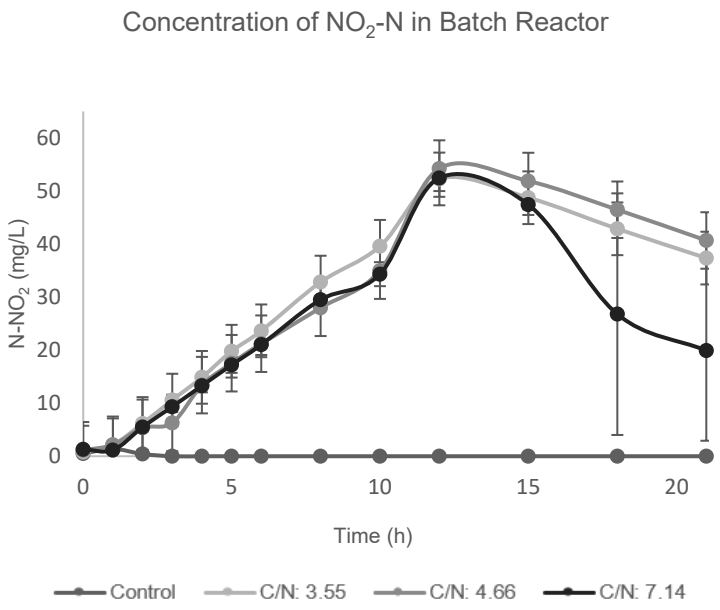

Figure 2. Nitrite concentration dynamics in batch reactor trials at different C:N ratios in freshwater.

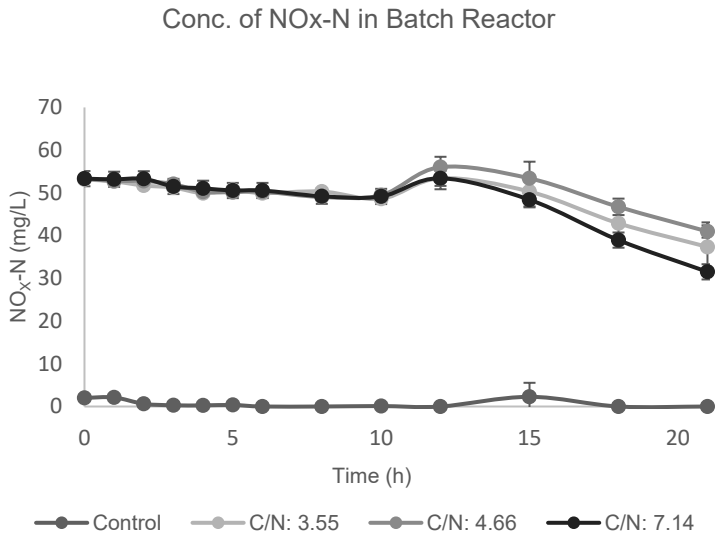

Figure 3. NOx concentration dynamics in batch reactor trials at different C:N ratios in freshwater.

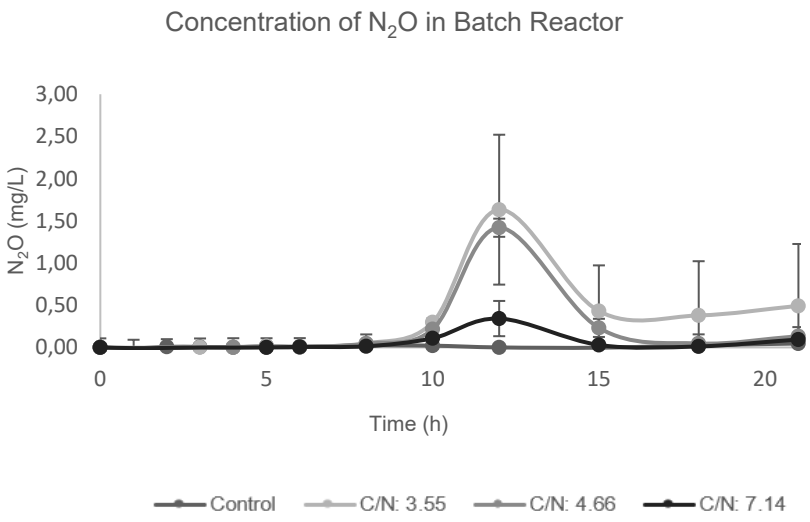

Figure 4. Nitrous oxide concentration dynamics in batch reactor trials at different C:N ratios in freshwater.

There was no difference in the removal rates of NO_3-N nor NO_2-N accumulation rates from the 4th to 10th hour. The concentration of NOx-N was decreasing slightly throughout the experiment for all treatments but there was no difference in the removal rates of NOx-N between treatments.

The reactors with 7.14 C:N ratio had a higher removal rate of NO_2-N from the 12th to 21st hour ($P = 0.02$). This was consistent with pH being higher for reactors with 7.14 C:N ratio at the end of the experimental period ($P = 0.032$). Lastly, the reactors with 7.14 C:N ratio had a numerically lower peak N_2O concentration when normalized for bacterial biomass ($P = 0.08$).

The decrease in nitrate and increase in nitrite concentration observed corresponds to the first step of the denitrification pathway (Equation 4). The reductions in this pathway are typically conducted by different microorganisms which are specialized to perform one or a few reduction reactions i.e. stepwise, rather than the whole reaction (Marchant et al., 2018).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad \text{(Equation 4)}$$

The amount of nitrate reduced was more than the nitrite accumulated and thus both nitrate and nitrite were reduced simultaneously. As nitrite reduction was 10 times slower than nitrate reduction, nitrite accumulation occurred until nitrate was depleted. This was observed, although with differences, when comparing the proportion of nitrate-nitrite reduction rates.

The substantially higher nitrate reduction rate compared to nitrite reduction explains the decrease in pH. The substantially higher reduction rate of nitrate produced more CO_2 which reduced the pH. This was initially not countered by the production of hydroxide ions in the 2nd step reaction due to the much slower nitrite reduction rate. The increase in pH was only observed when nitrate (Pan et al., 2012)was depleted, which stopped the 1st step reaction.

The depletion of nitrate/reduction of nitrite also coincided with the peak in N_2O concentration. This observation was reported by Adouani et al. (2014)), where an increase in N_2O concentration was also observed with the peak production of NO_2^-. This could be due to nitrite reductase consuming a higher fraction of electrons than nitrous reductase resulting in N_2O accumulation.

In this trial, the N_2O peak occurred when nitrite was almost completely reduced at the 5th hour. Nonetheless, the higher C:N ratio (i.e. 6.6) had a numerically lower N_2O concentration which is consistent with the findings in this trial (i.e. freshwater denitrifying bacteria). In addition, the highest C:N ratio also had a higher NO_2-N reduction rate, which suggests that the higher C:N ratio the more energy is available for the bacteria to complete the denitrification pathway (Equation 4).

C:N Batch Trial saltwater

NO_3^- was completely reduced at similar time in reactors containing C:N 2.7 and C:N 4. The reactor with a C:N 0.5 shows very slight or close to no decrease in NO_3^- over time, indicating there is not enough carbon to go through the process (Fig. 5).

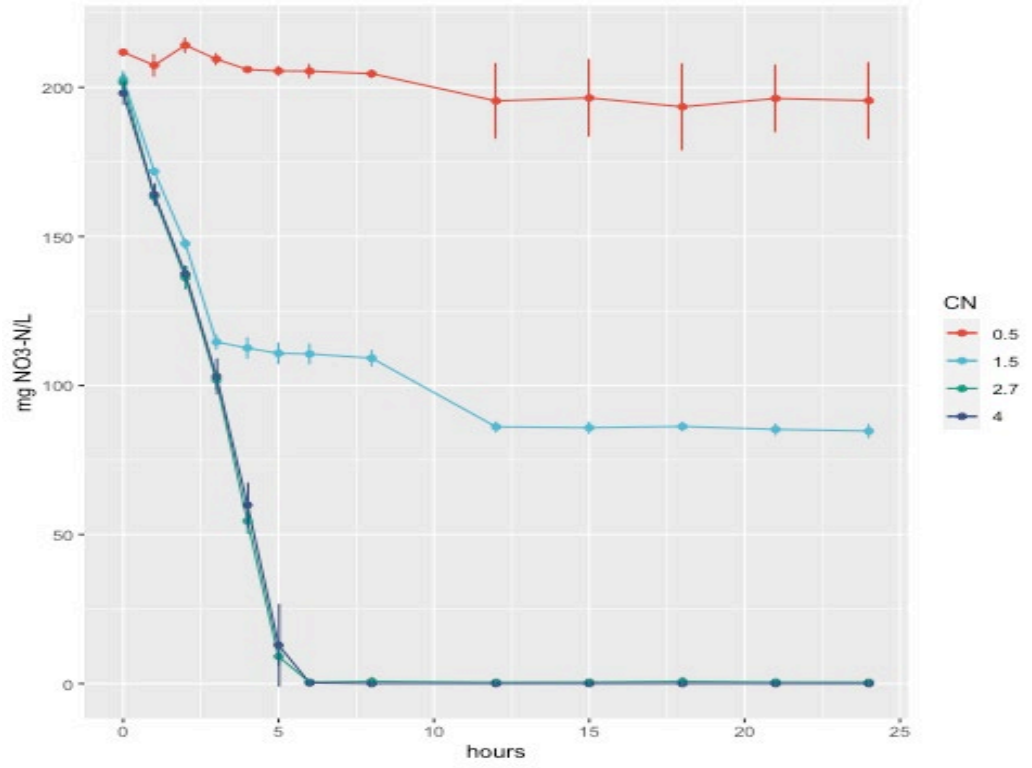

Figure 5. Nitrate concentration dynamics in batch reactor trials at different C:N ratios in saltwater.

Specific denitrification rates and volumetric denitrification rates (**Table 1**) reflect the effect of C:N on the denitrification process.

Table 1. Specific denitrification rate and volumetric denitrification capacity of NO_3^--N in reactors supplied with acetate and nitrate to reach listed C:N ratios.

NO_3—N C:N	Specific denitrification rates	Volumetric denitrification capacity
0.5	0 mg NO_3^--N / (h g biomass)	0 kg NO_3^--N / (m^3 reactor d)
1.5	1.8 mg NO_3^--N / (h g biomass)	0.23 kg NO_3^--N / (m^3 reactor d)
2.7	6.6 mg NO_3^--N / (h g biomass)	0.81 kg NO_3^--N / (m^3 reactor d)
4	6 mg NO_3^--N / (h g biomass)	0.79 kg NO_3^--N / (m^3 reactor d)

At a C:N 0.5 there is no production of NO_2^--N over time as no denitrification process occurred due to the low C:N ratio. C:N 1.5, C:N 2.7 and C:N 4 show an increase in NO_2^--N over time in the first 3 hours of the process where posteriorly stabilizes (Fig. 6).

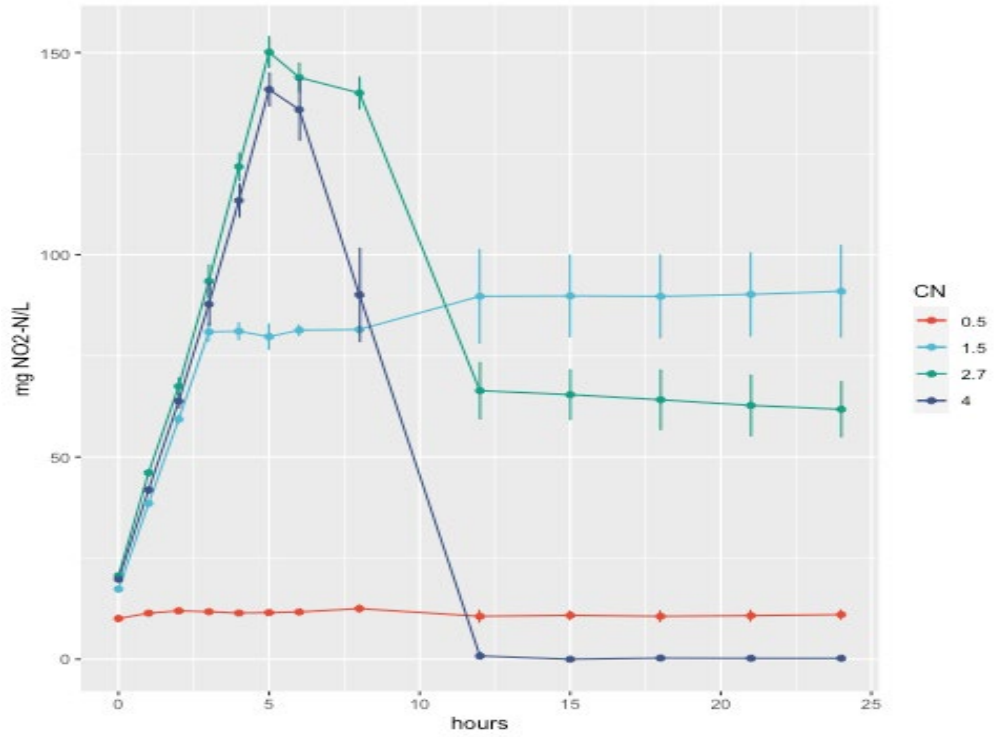

Figure 6. Nitrite concentration dynamics in batch reactor trials at different C:N ratios in saltwater.

A C:N 1.5 shows an increase in dissolved N_2O from 4 hours to 15 hours since the start of the process, reaching a max concentration of 3.6±0.8 mg/L N_2O at 15 hours followed by a continuous decrease. At a C:N of 2.7 an increase in dissolved N_2O is seen from 7 to 21 hours, reaching a max concentration of 2.6±1 mg/L N_2O at 21 hours followed by a decrease in the last measurement (24 hours).

N_2O production is not only largest in C:N 1.5 but the production also reaches a max faster than C:N 2.7 (Fig. 7). When normalized and calculated into specific production rate C:N 1.5 produces double the amount of N_2O per g biomass as the reactors with C:N 2.7.

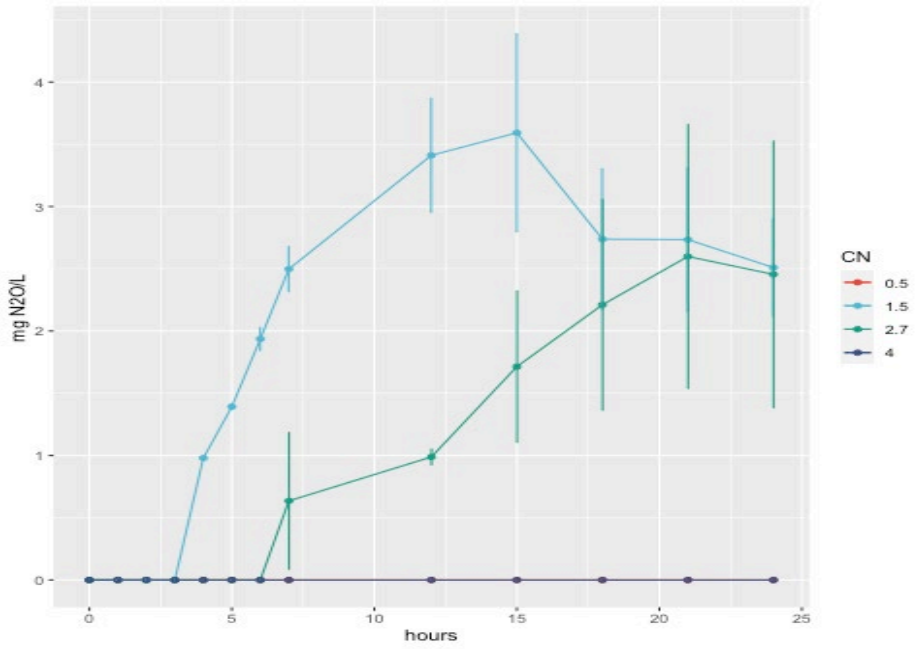

Figure 7. Nitrous oxide concentration dynamics in batch reactor trials at different C:N ratios in saltwater.

Table 2. Specific denitrification rate and volumetric denitrification capacity of NO_3^--N in reactors supplied with acetate and nitrate to reach listed C:N ratios.

	N_2O	
C:N	**Specific production rate**	**Volumetric production capacity**
0.5	0 mgN_2O / (h g biomass)	0 gN_2O / (m^3 reactor d)
1.5	0.04 mgN_2O / (h g biomass)	5.1 gN_2O / (m^3 reactor d)
2.7	0.02 mgN_2O / (h g biomass)	2.5 gN_2O / (m^3 reactor d)
4	0 mgN_2O / (h g biomass)	0 gN_2O / (m^3 reactor d)

A reactor containing C:N 1.5 can produce 5.1 g N_2O/m^3 reactor/d, indicating the amount potentially produced by a mismanaged process operation.

In all reactors no H_2S was detected. This was also the case, after acidifying samples to pH = 2 to ensure H2S <-> HS^- equilibrium shifted towards H_2S. Meaning C:N ratios at 4 or below do not have excessive carbon as electron donors after denitrification.

Based on the stoichiometry, a COD/N ratio of 3 for acetate would lead to complete denitrification (Adouani et al., 2014). However, different parameters enter into account as presence of oxygen in the water or an incomplete development of the different bacteria associated to the 4-step reduction from NO_3^- into N_2 gas. As seen in the experiments in fresh and saltwater, the increase in C:N affects the process kinetics although showing a nitrate reduction. However, even though in the freshwater experiment the C:N was higher, the second step there is an accumulation of NO_2^- This could be explained by the difference in the biomass utilized for the evaluation, while in the

1.7.2 General remarks C:N in denitrification

The trials at different C:N ratio and salinity show how the denitrification process is strongly regulated by the C:N ratio at the production of intermediates as NO_2^- and N_2O. Both compounds impose negative effects as NO_2^- is toxic to aquatic life at low concentrations, while N_2O is a strong greenhouse gas (300 times CO_2). However, there are other factors that can regulate the accumulation of these compounds such as presence of oxygen which will affect the nitrite reductase impairing nitrate reduction or a non-mature bacterial population lacking these organisms as they tend to grow at a slower pace than nitrate reducing bacteria. Furthermore, if not enough carbon sources are present the process is interrupted creating the accumulation of nitrous oxide.

In the saltwater trial, the xCOD concentration used was 4.8±0.3 g/L which was 2.8 times higher than the concentration used in this trial i.e. at 1.73±0.3 g/L. Furthermore, the bacterial biomass utilized in the saltwater trials was more mature than in the freshwater one. As such, the lack of differences observed in the different C/N ratio treatments is likely due to low bacterial concentration and immature bacterial population with poor denitrification efficiency.

In general a low C:N results in intermediate products as NO_2^- and N_2O and (C:N 1.5 and C:N 2.7). A very low C:N (<2) does not result in any intermediate reactions, as no denitrification and reduction of NO_3^- occurs. No production of H_2S was found in the saltwater and freshwater trials, contrary to the hypothesis that at a higher C:N ratio the production of H_2S is facilitated by extra carbon availability and presence of sulfate-reducing-bacteria which will utilize sulfate as terminal electron acceptor when NO_3^- is not available. It is important to bear in mind that above a pH 6.6 HS^- is the dominant species and below pH 6.6 H_2S becomes dominant. In this sense a full denitrification process will add alkalinity to the water raising the pH of the solution up to 9, therefore all sulfide fraction is found as HS^-. Even though in this experiment no H_2S was found even after acidification of samples to pH 2 is important to have in mind that a high C:N (over 7) can lead to extra carbon availability and thus initiation of H_2S formation. Reason why it is recommended not to remove all nitrate from the water.

Under these conditions it is concluded that a marine RAS end of pipe denitrification reactor should not go below C:N = 4 to keep optimal denitrification conditions. Going higher than C:N =4 is not recommended because it is more economical not to use excessive acetate and the risk of producing H_2S might increase.

1.8 Denitrification systems

1.8.1 Fed-batch reactor system design and set-up

Flocculating capacity bacteria was conditioned in three 10L Pyrex® media bottles with a concentration of 330 NO_3^--N mg/L and sodium acetate to reach a COD:NO_3^--N of 6. This allowed the bacteria to mature i.e. more established/ diverse bacterial biome and greater density/ larger flocs. To calculate the settling time required for the FBR, batch settling tests as described in (Koo, 2009) were also conducted to determine the flocs settling velocity as a function of bacteria concentration (Fig. 8 and 9). The settling time was verified again prior

to inoculation of the bacteria in the FBR and was determined that a 20-minute settling time would be sufficient for the cultivated flocculent bacteria.

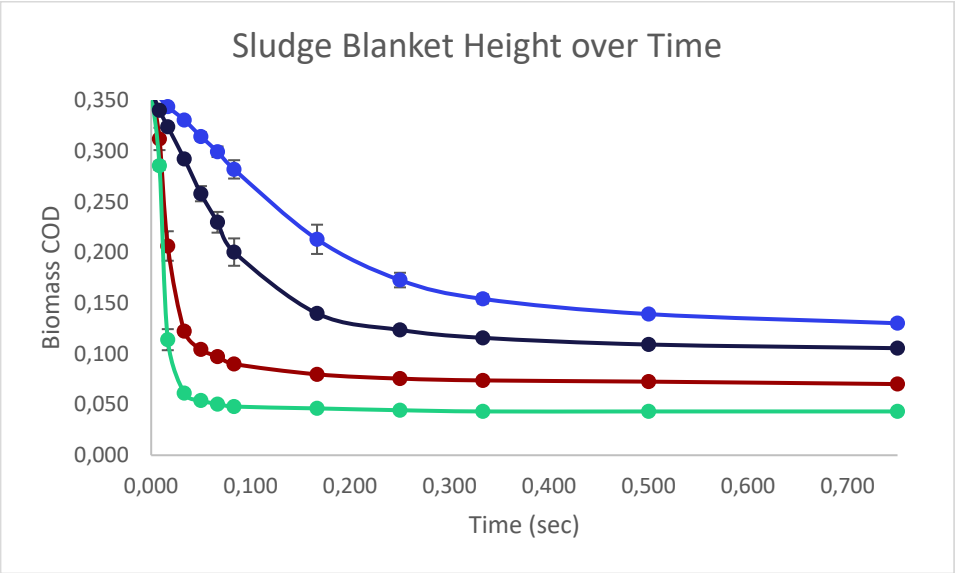

Figure 8. Settleability capacity of flocculant bacteria through time.

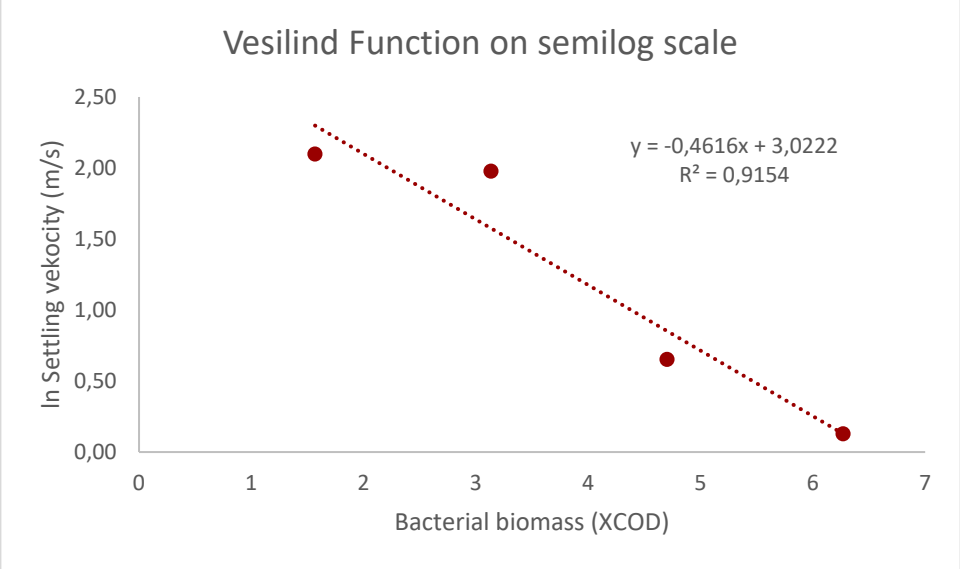

Figure 9. Vesilind function of settling velocity developed with flocculant bacteria.

A freshwater RAS with an intensity of 1.63 m^3 make up water/kg feed was connected to the FBRs via the sump as per Figure 10. The RAS had approximately 50 kg of rainbow trout fed at 1% body weight per day i.e. 500g. The fish were fed EFICO E 920 Adv, 4.5mm, formulated by BioMar. The total system volume of the RAS was 2960 L with a cubic rearing tank of 2400 L, 2 moving bed bioreactors (MBBR) of 60 L each, a sump of 400 L and a protein skimmer of 40 L.

Abiotic requirements of the species were set and monitored daily before morning feeding and any system management. The temperature, oxygen and pH were measured with a multiparameter (Hach HQ40d, Hach Lange, Germany) while NH_4^+-N, NO_3^--N, NO_2^--N was measured with respective kits (Aquamerck 1.11118.0001; Mercoquant 1.10020, Merck KGaA, Darmstadt, Germany). All measurements were taken from the settling tank. Sodium bicarbonate was added to maintain the pH when necessary.

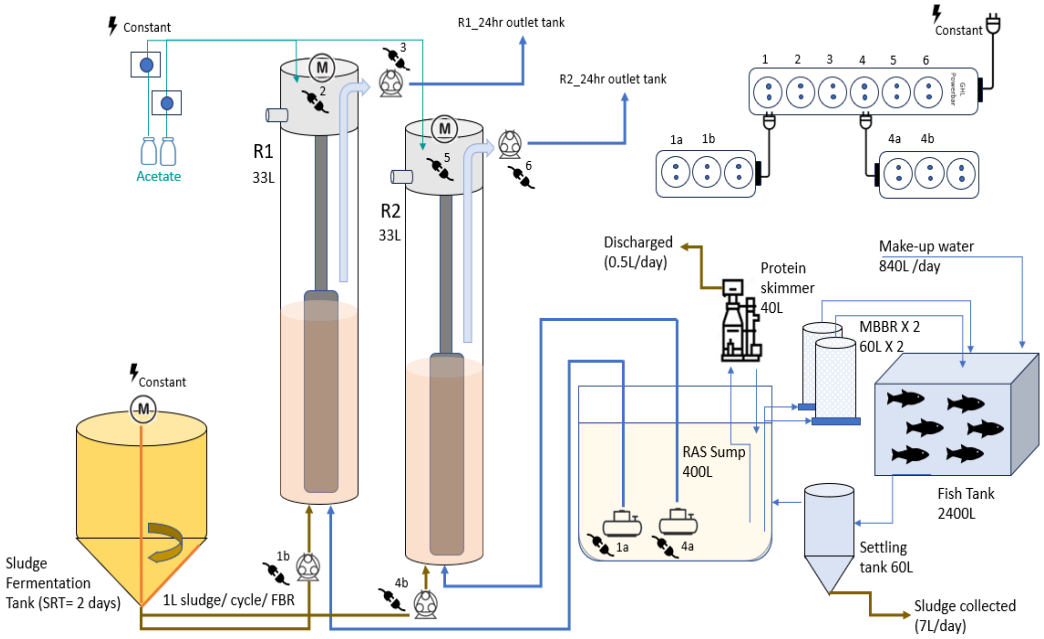

Figure 10. Experimental layout showing the connections between the RAS and FBR.

The two FBRs were 33 L each, with each connected to the RAS sump via a submersible pump (AquaMedic DC-Runner 5.2), 2 peristaltic pumps (Longer Pump YZ1515x and Longer Pump BT100-2J) and a dosing pump (GHL Doser 2.2 Stand Alone). One peristaltic pump was connected to the sludge fermentation tank to supply 1L of sludge per filling cycle while the other was connected to the outlet of the FBR to discharge 11L of supernatant after each settling phase. The dosing pump was connected to a bottle containing 240g acetate/L set to dose at each filling cycle to make a C:N ratio of 6. Each FBR was fitted with an emergency overflow outlet and paddle mixer connected to a gear-box motor (HJP37RIC150i-Z6001, 30 rpm) at the top of the reactor. All pumps, motors and dosing pumps were operated automatically via a programmed power socket (GHL Powerbar 6E-PAB) with a fill, settling and discharge time of 3, 20 and 6 minutes respectively. The motors were operated at a predetermined HRT (i.e. 7.5, 5.5, 3.5 hours) until the daily individually pooled NO_3^--N concentration of the FBR outflow remained steady within a 20% variation for 4 days i.e. steady state.

Prior to the start of the experiment, nitrate concentration of the RAS was measured to be steady at 20 NO_3^--N mg/L for a week. xCOD was used as an indicator of biomass concentration (Henze et al., 1997a) and both reactors were inoculated with 575 xCOD mg/L of flocculent bacteria with a settled volume index (SVI) of 121.7 mg/L. Throughout the experi-

mental period, approximately 7 L of sludge was collected from the settler daily and transferred into the sludge fermentation tank with a minimum solid retention time of 2 days before pumped into the FBR.

Pooled samples of a 24-hour cycle were collected three times a week to monitor denitrification performances. 100 mL sample for Total chemical oxygen demand (TCOD), Total Nitrogen (TN), Total Phosphorus (TP), and two 15 mL sample for VFAs, SCOD, NH_4^+-N , NO_3^--N, NO_2^-N, PO_4^{3-}-P and SO_4^--S (anions) were collected from each FBR outlet tank, sludge fermentation tank, RAS sump and fish tank. Samples for TCOD, TN, TP, VFAs and SCOD were preserved by adding 1% v/v sulfuric acid. Sample for anions were filtered through 0.2 µm syringe filters (Filtropur S, SARSTEDT, Germany). All the samples were maintained at 4 °C until analysis. pH was also measured for all samples immediately after collection using the handheld meter (Hach HQ40d, Hach Lange, Germany) which was calibrated weekly.

1.8.2 Activated sludge at a commercial scale RAS facility

Danish Salmon A/S (1500MT/year) treats approximately 20% of their overflow water with a denitrification process prior to discharging the outflow to the sea. The denitrification tank or process tank with a volume of 175 m^3) receives streams from three other tanks (Fig. 11). The clean tank containing the water from the overflow of the fish tanks resulting from water exchanges deliver 19.9 m^3/h into the process tank, 2) the sludge tank containing the backwash of the drum filters and biofilters delivers 9.6 m^3/h into the process tank and 3) the clarifier with a volume of 180 m^3, where the activated sludge is settled and a fraction (8.7 m3/ h) recirculated back to the process tank while the other fraction is discharged into the sea. Based on these flows, the process tank has an HRT of 4.6 hours while the sludge tank has an HRT of 29.5 hours.

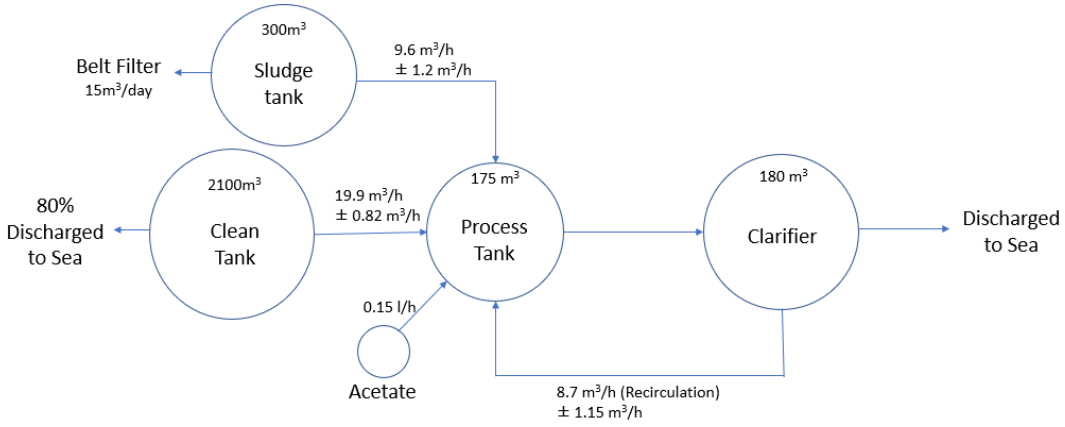

Figure 11. Schematic of denitrification process with respective flows from each tank at Danish Salmon A/S, Hirtshals, Denmark.

Automatic portable samplers (Glacier ISCO, Teledyne, USA) were used to take samples from the clean tank, sludge tank, clarifier, and process tank. Hourly samples taken over 24 hours were pooled and collected 3 times a week to monitor denitrification performance.

Samples for TCOD, VFA, sCOD and anions were treated and stored as previously explained. To retain the gases, samples for pH, redox, DO, H_2S and N_2O were collected using a 50 mL tube and immediately analyzed (i.e., separate from the pooled sample). Samples were collected over a course of 64 days. Corresponding flow rates and feeding data recorded by the farm were also obtained for the observation period. The farm during the evaluation period was feeding an average of 3,912 ± 331 kg/ day. The flow rates with their corresponding standard deviation are indicated in Fig. 11.

1.8.3 Upflow denitrification sludge blanket

An upflow denitrification sludge blanket reactor (UASB) (Fig. 12) was built and evaluated on the nitrate and nitrite removal capacity. The sludge was conditioned according to Letelier and Herreros (2019) during three weeks in a fed-batch condition. After that period the flocculent bacteria showed a progressive reduction in the SVI numbers from 34.7 ml/g to 15.4 mL/g, showing good settleable conditions.

The reactor operates under an upflow configuration where a peristaltic pump delivers the water to the bottom of the reactor at a speed of 1.2 m/h. A recirculation pump installed at 3/4 of the total height of the reactor with the purpose of recycling back the water with the compound to be treated. The objective is to increase the contact time between the bacteria and the substrate for denitrification (carbon and nitrogen). At the top end of the reactor water is discharged.

For the evaluation the reactor was setup as batch configuration where nitrate at 150 NO_3-N mg/L.

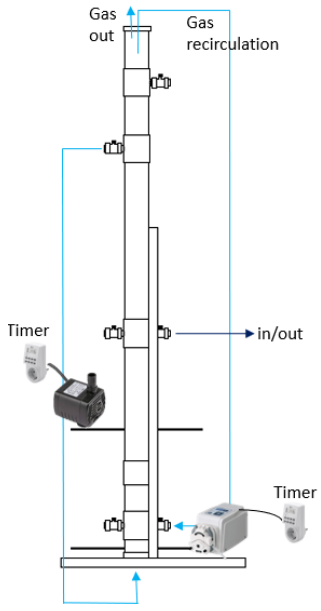

Figure 12. UASB reactor.

1.9 Sample analysis

Anions (nitrate, nitrite, phosphate and sulfate) and VFAs were analyzed with an ion chromatography (930 Compact IC Flex 1 with a Metrosep A Supp 7- 250/4.0 column combined with a 887 Professional UV/VIS detector Metrohm, Sweden) with 0.1 M H_2SO_4 as suppressor and 3.6 mM Na_2CO_3 as eluent. TCOD and sCOD were determined using digestion vials (LCK 314, 514, and 1414, Hach Lange, Germany). pH and temperature were monitored using a portable meter (Hach HQ40d, Hach Lange, Germany). The 6 days were selected to give a representative sample of TCOD and SCOD values throughout the observation period. VFAs were determined using 881 Compact IC Pro (Metrohm, Sweden) combined with an 887 Professional UV/VIS Detector (Metrohm, Sweden). 0.1 M LiCl as suppressor and 0.5mM HClO4 as eluent were used. The mobile phase lasted 1 hour with a flow rate of 0.25 mL/min at 35 °C. N_2O and H_2S measurements were performed with a SULF-500-210675 and N_2O-R-207746 respectively (Unisense, Denmark), and measurements were recorded with the software SensorTrace Logger. In this report, TCOD and SCOD were only analyzed for 6 of the 19 sampling days.

1.10 Data analysis and equations

All calculations, based on the following equations were done with Excel for Microsoft 365. Statistical analysis of one-way type 2 ANOVA to calculate the significant ($p<0.05$) between treatments were done with Rstudio (version 1.4.1717). Assumptions for ANOVA, i.e. equal variance and normal distribution were checked with Rstudio using Levene's test and Shapiro-Wilk test. Graphs were made using both Excel and Rstudio.

$$\boldsymbol{Specific\ denitrification\ rate}\ (\boldsymbol{mg/L/hour/\ xCOD}) = \frac{\boldsymbol{\Delta\ in\ concentration}}{\boldsymbol{time}} \times \frac{\boldsymbol{1}}{\boldsymbol{xCOD}} \quad \text{(Equation 5)}$$

Bacterial biomass $\boldsymbol{xCOD = TCOD - SCOD}$ (Equation 6)

$$\boldsymbol{Sludge\ Volume\ Index}\ (\boldsymbol{SVI}) = \frac{\textbf{settled sludge volume}\ (\boldsymbol{measured\ at\ 30\ minutes})}{\textbf{xCOD}} \quad \text{(Equation 7)}$$

$$\boldsymbol{Mass\ load}\ (\boldsymbol{kg/day}) = \boldsymbol{Concentration}\ (\boldsymbol{kg/m^3}) \times \boldsymbol{Flow}\ (\boldsymbol{m^3/day}) \quad \text{(Equation 8)}$$

$$\boldsymbol{Efficiency}\ (\%) = \frac{\boldsymbol{Quantity\ Treated}}{\boldsymbol{Quantity\ supplied\ into\ CV}} \quad \text{(Equation 9)}$$

Control volume (CV) is defined as indicated by the box in the schematic below (Fig. 13):

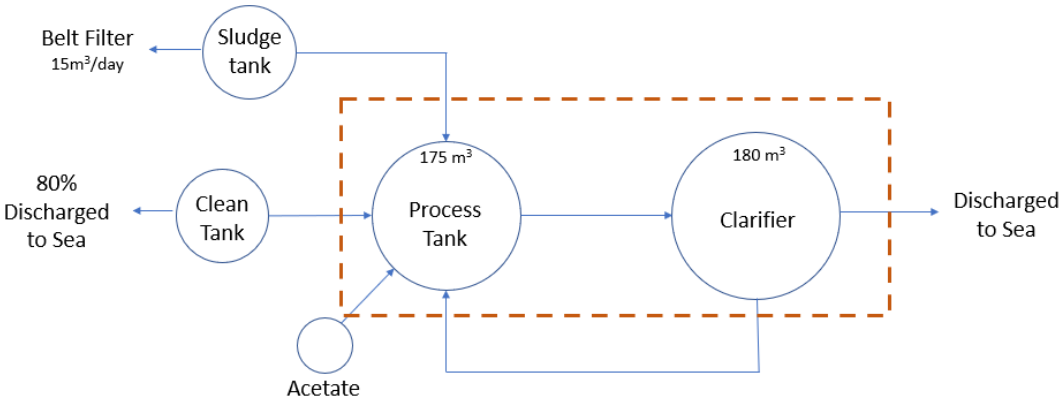

Figure 13. Control volume for Danish Salmon denitrification system.

$$\%\ \boldsymbol{of\ treatment} = \frac{\boldsymbol{Quantity\ Treated}}{\boldsymbol{Quantity\ Treated + Quantity\ Discharged\ to\ Sea}} \quad \text{(Equation 10)}$$

$$\boldsymbol{N_2O\ emission\ rate} = \boldsymbol{N_2O\ mass\ transfer\ coefficient} \times \left[\boldsymbol{N_2O\ concentration} - \frac{\boldsymbol{N_2O\ concentration\ in\ air\ equilibrium}}{\boldsymbol{Henrys\ Constant}}\right] \quad \text{(Equation 11)}$$

Values used for calculations: N_2O mass transfer coefficient = 3, N_2O concentration in air equilibrium = 0.0003, Henrys Constant = 0.0247 (Unisense Environment A/S, 2022).

1.11 Results and discussion FBR trials

The average water quality parameter from the FBR evaluation is summarized in Table 3.

Table 3. The average ± standard deviation of water quality parameters of the fish tank, corresponding RAS Sump, sludge tank, and 24-hour pooled samples from the respective feed batch reactors.

Parameter	Fish Tank	RAS Sump	Sludge Tank	FBR 1	FBR 2
pH	7.14 ± 0.08	7.12 ± 0.08	6.79 ± 0.14	7.65 ± 0.09	7.72 ± 0.21
TCOD [mg/L]	41.1 ± 4.5	41.38 ± 3.48	4945 ± 1550	1110 ± 364	758 ± 256
sCOD [mg/L]	24.7 ± 1.31	24.6 ± 1.58	710 ± 370	1021 ± 310	696 ± 249
xCOD [mg/L]	16.4 ± 5.1	16.8 ± 4.96	4235 ± 1182	88.5 ± 66.8	61.8 ± 33.4
NO_3^--N [mg/L]	24.1 ± 1.74	23.6 ± 1.61	2.21 ± 3.55	0.88 ±1.42	0.13 ± 0.04
NO_2^--N [mg/L]	0.38 ± 0.09	0.36 ± 0.01	0.01 ± 0.00	1.37 ± 2.71	0.01 ± 0.00
NH_4^+-N [mg/L]	0.61 ±0.01	0.69 ± 0.01	16.9 ± 17.7	0.78 ± 0.63	0.64 ± 0.58
PO_4^{3-}-P [mg/L]	1.72 ± 0.12	1.96 ± 0.59	21.66 ± 12.25	2.54 ± 1.4	4.13 ± 3.35
Acetate [mg/L]	0.66 ± 1.31	0 ± 0	292.6 ± 152.1	989 ± 316	640 ± 257
Propionate [mg/L]	0 ± 0	0 ± 0	109 ± 83.78	4.05 ± 2.86	3.47 ± 2.81
Formate [mg/L]	0 ± 0	0 ± 0	7.12 ± 4.76	4.55 ± 5.25	4.53 ± 5.23
Butyrate [mg/L]	0 ± 0	0 ± 0	15.73 ± 11	2.57 ± 1.75	2.30 ± 1.55
VFA CODeq [mg/L]	0.70 ± 1.41	0 ± 0	519.5 ± 311	1071 ± 346	696.2 ± 279

As shown in Figure 14, the higher nitrate and nitrite concentration were observed only in the outlet of FBR 1 at the start of the experiment (Day 1). For the subsequent samplings, there was no nitrate and nitrite observed in the outlet of both FBRs. As such, it was deemed that steady state was reached on 19 July. Both FBRs show that there was increasing concentration of NH_4^+-N and PO_4^{3-}-P which was similarly observed in the sludge fermentation tank (Fig. 14, 15 and 16).

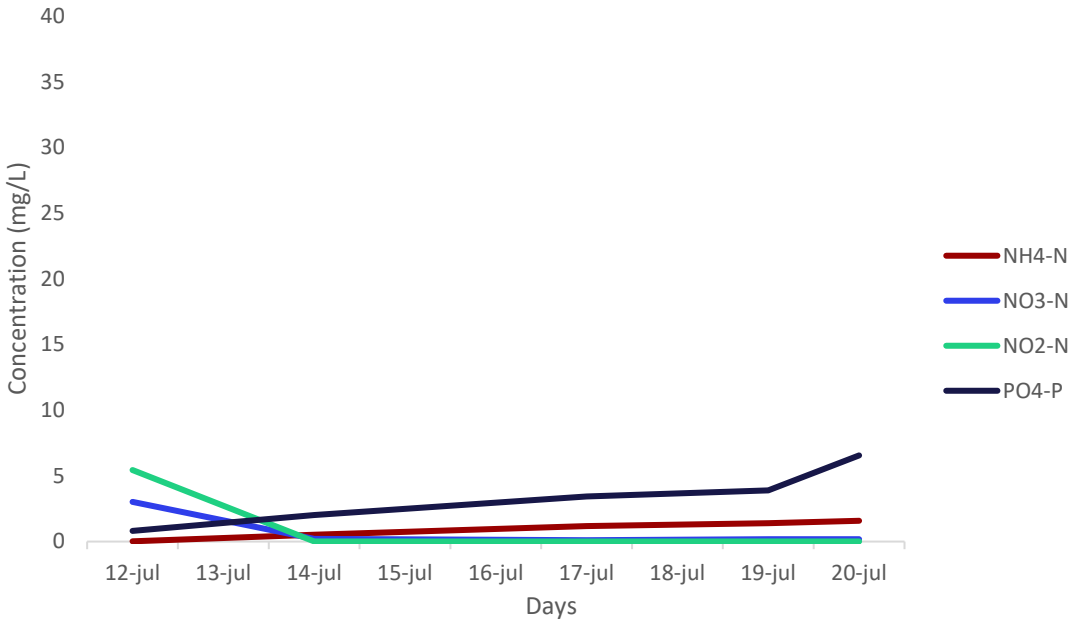

Figure 14. Nutrient dynamics in the effluent of fed batch reactor 1.

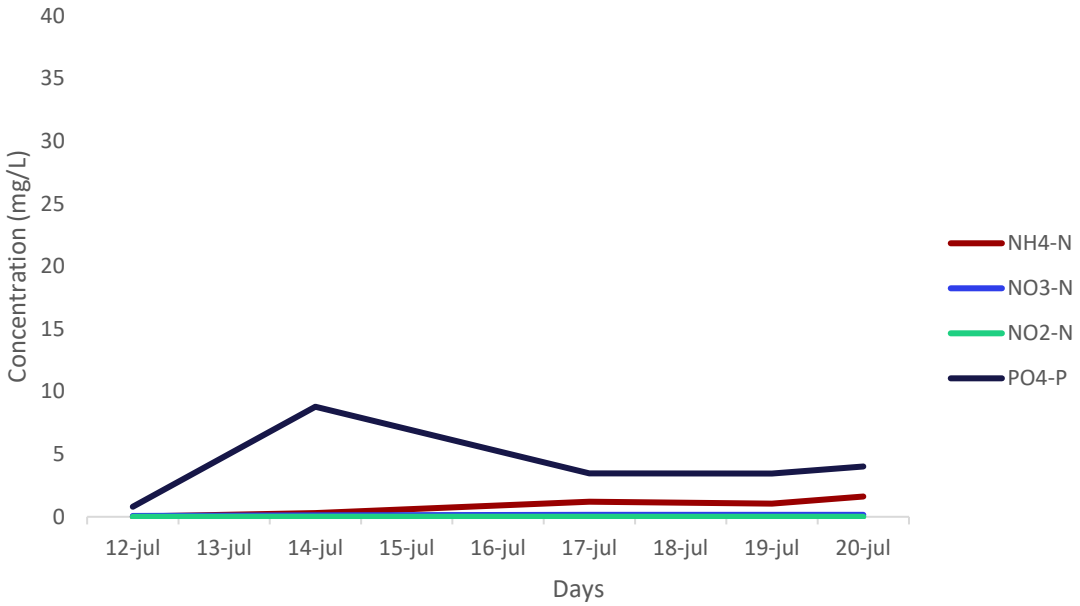

Figure 15. Nutrient dynamics in the effluent of fed batch reactor 2.

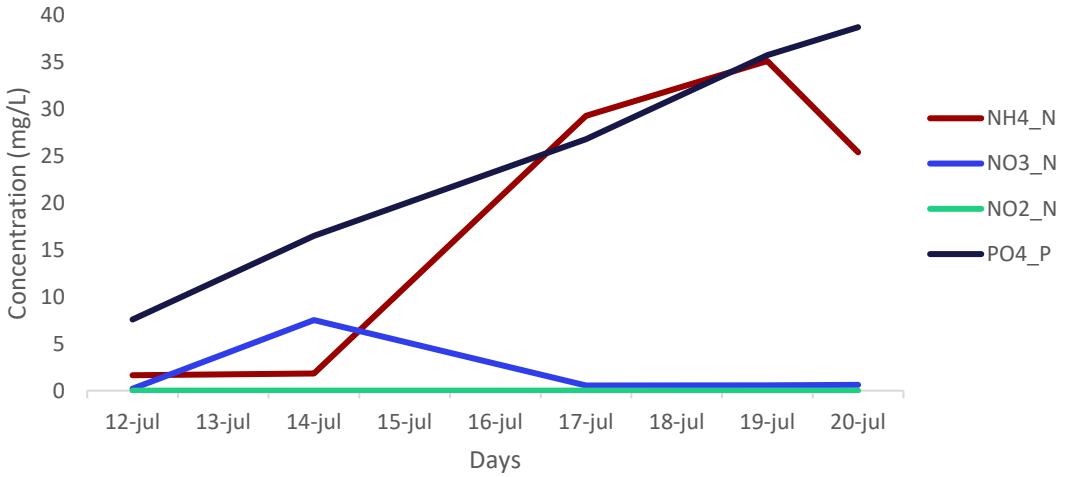

Figure 16. Nutrient dynamics in the effluent of sludge fermentation tank.

The 24-hour sampling regime on 20 July (3 cycles) shows that the outflow of both FBRs had a consistent level of nutrient and COD outflow (Table 4).

Table 4. Water quality parameters of the 24-hour sampling regime for the FBR outlets.

Parameters	FBR 1	FBR 2
NH_4^+-N (mg/L)	2.0 ± 0.42	1.98 ± 0.33
NO_2^--N (mg/L)	0.01 ± 0	0.01 ± 0
NO_3^--N (mg/L)	0 ± 0	0 ± 0
PO_4^{3-}-P (mg/L)	4.82 ± 1.47	4.10 ± 0.18
TCOD (mg/L)	1155 ± 19.5	1009 ± 40.4
$_S$COD (mg/L)	1066 ±5.85	921.7 ± 56.7
XCOD (mg/L)	89 ± 25.4	87.3 ± 16.3
VFA CODeq (mg/L)	1122 ± 11	964.9 ± 52.3

The 24-hour sampling regime showed that high denitrification efficiency in terms of nitrate removal was achieved for both FBRs. However, low efficiency was achieved for COD (Table 5).

Table 5. Efficiencies of the FBRs based on mass loading calculations.

Parameter	FBR 1 Efficiency (%)	FBR 2 Efficiency (%)
NH_4^+-N (mg/L)	30.54	31.16
NO_2^--N (mg/L)	98.41	98.58
NO_3^--N (mg/L)	100	100
Dissolved N	92.58	92.65
PO_4^{3-}-P (mg/L)	-38.32	-16.28
TCOD (mg/L)	30.46	37.47
$_S$COD (mg/L)	9.14	18.13
XCOD (mg/L)	81.8	82.1
VFA CODeq (mg/L)	0.4	10.6

The SCOD:N ratio supplied into FBR 1 and 2 is 48 and 46 respectively. Of which, 9% of the SCOD came from the sludge fermentation tank, the rest was supplied as acetate and unfortunately there was an overdose as initially estimated due to wrong calibration of the peristaltic pump delivering a significantly higher C:N than calculated. This resulted in 8.7 times more acetate being supplied than necessary, which resulted in such a poor treatment efficiency of SCOD i.e. 9-18%. In addition, there was also a high concentration of SCOD in the outflow of the FBRs ranging from 12 – 10 g SCOD/L. All in all, a high denitrification efficiency was achieved.

The hydrolysis of sludge also provided quite a substantive amount of SCOD, with sCOD making up 17% of the TCOD of which 73% of the SCOD was from VFAs. This is in line with the findings from (Suhr et al., 2013) where 75% of the SCOD constituted VFAs. This meant that 1L of sludge already provided for a SCOD/N ratio of 4.4. Meriac et al. (2014) showed that a biodegradable COD (from faecal waste) to nitrogen ratio of 4.4 was required for complete denitrification. This indicates that enough endogenous carbon was provided through the organic matter produced in the RAS. The amount of SCOD provided from the sludge fermentation tank is in line with estimations from Model Trout Farm in Denmark which could produce a COD/N ratio of 5.2 – 6.7 ((Jokumsen and Svendsen, 2010).

Due to the high SCOD/N ratio, denitrification efficiency was 100%. This is in line with research showing that at an optimal COD/N ratio of 4.6 with sodium acetate would achieve a nitrate removal rate of 99% (Li et al., 2008). In addition, it was also shown that sodium acetate was the optimal carbon source to suppress N_2O emissions. This suggests that we could further reduce the amount of acetate by 23% i.e. from a COD/N ratio of 6 to 4.3.

The start-up time of the FBR was also relatively fast with all nitrate and nitrite removed on day 4 of the FBR set up. FBR 2, showed that all nitrate and nitrite was removed on day 1 of the FBR setup and the reason why FBR 1 did not perform equally well was due to an issue with the paddle mixer. As such, the denitrifying bacteria did not have sufficient contact time with the substrate to complete the denitrification process. The high nitrite values in the outlet of FBR 1 on day 1 also supports that incomplete denitrification occurred. Nonetheless, the results indicate that the bacteria in the FBR quickly adjusted from the 3 X 10L reactor to the 2 X 33L reactor and operated effectively in its expected capacity.

The poor efficiency of NH_4^+-N and production of PO_4^{3-}-P in the FBRs could be attributed to the hydrolysis of sludge and the breaking down of sludge by bacteria. This also corresponds to the increasing NH_4^+-N and PO_4^{3-}-P concentrations in the sludge tank (Fig.16). Due to the high SCOD/N ratio, it is also possible that some of the nitrate was converted to NH_4^+-N via dissimilatory nitrate reduction (van Rijn, 2013).

XCOD treatment efficiency in both reactors was relatively high which meant most of the bacterial biomass supplied by the sludge was retained in the FBRs. This showed that the settling time design was sufficient to retain most of the bacterial biomass in the FBR despite the relatively high starting SVI. Nonetheless, as the activated sludge adapts to the new settling time of 20 minutes, the reduction of settling time by 33% will likely improve (i.e. decrease) the SVI.

The mass loading calculations were conducted based on the average flow measurements conducted prior to the actual operation of the FBR. While the FBRs were operating well in the first 2 weeks i.e. for HRT 7.5, there were subsequently issues with flows i.e. overflowing, unequal water heights in the reactors and undersupply of effluent to the FBR. As such, the mass loading calculation, which depends greatly on the volume inflow and outflow could be significantly affected by these issues. Thus, mechanisms to record the outflow of the FBR could be installed to have a more accurate understanding of the FBR performance. Nonetheless, this evaluation has shown that the FBR is effective and efficient in the removal of nitrate and nitrite with a small footprint.

1.12 Results and discussion of Danish Salmon activated sludge system

The average characteristics of the water from the four tanks involved in the denitrification process are reflected in Table 6.

Table 6. The average ± standard deviation of water quality parameters of the clean, sludge, denitrification (herein referred to as process) and clarifier tank at Danish Salmon A/S, Denmark.

Parameter	Clean Tank	Sludge Tank	Process Tank	Clarifier
pH	7.3 ± 0.13	7.2 ± 0.2	7.0 ± 0.2	7.1 ± 0.2
Redox [mV]	27 ± 28	27 ± 27	15 ± 30	28 ± 29
DO [mg/L]	8.1 ± 0.8	3.1 ± 2.6	1.4 ± 2.3	6.5 ± 2.2
TCOD [mg/L]	113 ± 83	1758 ± 815	5174 ± 2262	114 ± 58
sCOD [mg/L]	32 ± 13	68.7 ± 22	367.3 ± 743	43.5 ± 19
xCOD [mg/L]	81 ± 81	1690 ± 829	4807 ± 2452	70 ± 47
SVI [mg/L]	19 ± 36	50.8 ± 24	90.5 ± 74	44.7 ± 44
NO_3^--N [mg/L]	43.5 ± 12	26.7 ± 8.8	0.4 ± 0.6	0.5 ± 0.5
NO_2^--N [mg/L]	0.84 ± 0.4	2.56 ± 1.2	0.45 ± 0.6	1.72 ± 1.4
NH_4^+-N [mg/L]	1.3 ± 0.85	1.17 ± 2.1	0.67 ± 1.2	0.32 ± 0.4
TDN [mg /L]	45.7 ± 12	30.4 ± 8.4	1.5 ± 1.3	2.5 ± 1.7
PO_4^{3-}-P [mg/L]	1.6 ± 0.77	2.1 ± 0.74	1.56 ± 1.8	0.51 ± 0.8
Acetate [mg/L]	1.13 ± 1.8	4.78 ± 3.9	135.7 ± 504	3.96 ± 13.8
Propionate [mg/L]	0.09 ± 0.4	0.28 ± 0.8	2.46 ± 3.5	0 ± 0
Formate [mg/L]	0.3 ± 1.2	0.52 ± 2.3	2.38 ± 4.8	0 ± 0

Based on an average daily feeding rate of 3,912 kg/day, the production model estimated that 1,152 kg of TCOD would be produced daily. However, only 674 kg of TCOD is discharged and entered the process tank (58% of TCOD produced). Using the average overflow to the sludge tank, the model predicts an estimated daily TCOD outlet concentration of 5,032 mg/L. The fluctuations in TCOD and SCOD values over the observation period are in Figure 17.

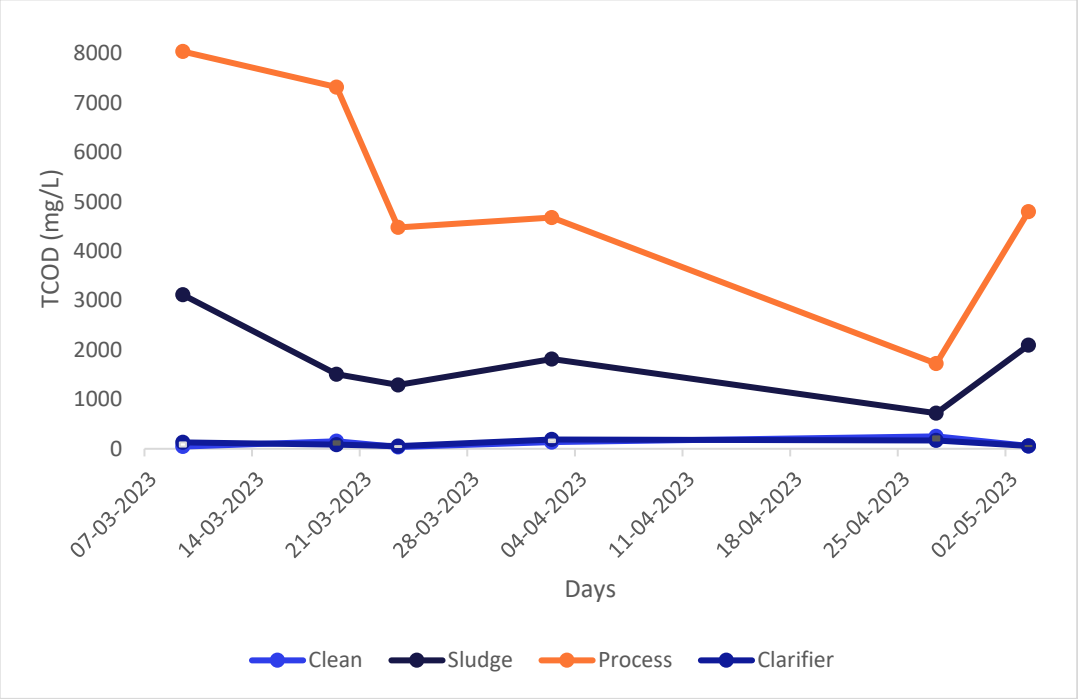

Figure 17. Total COD concentrations measured in the sludge tank of Danish Salmon A/S.

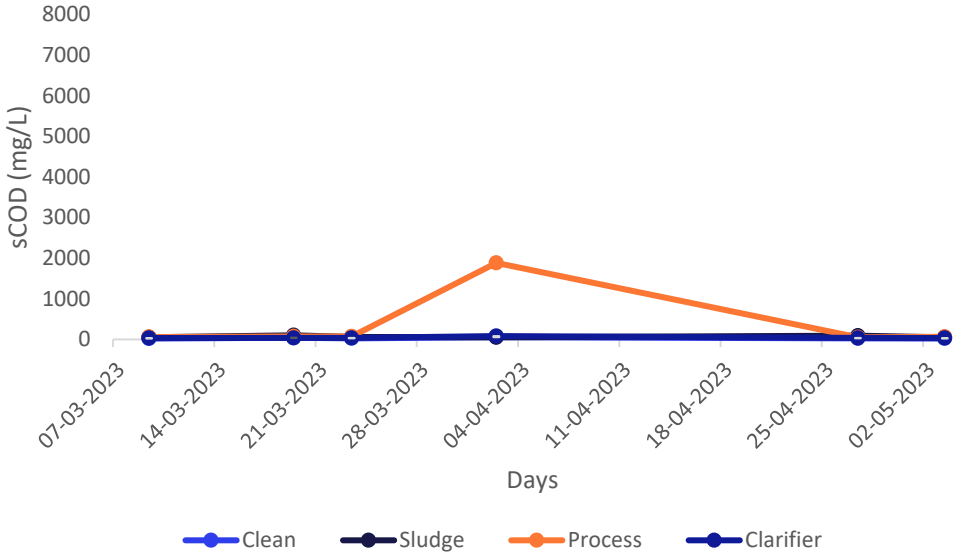

Figure 18. Soluble COD concentrations measured in the sludge tank of Danish Salmon A/S.

An abnormally high SCOD value was observed in the process tank on 2 April 2023. The SCOD concentration was 1,884 mg/ L and was 29 times higher than the average of the SCOD concentrations measured from the other 5 days. This is consistent with the acetate concentration measured on 2 April, which was 2,213 mg acetate/ L.

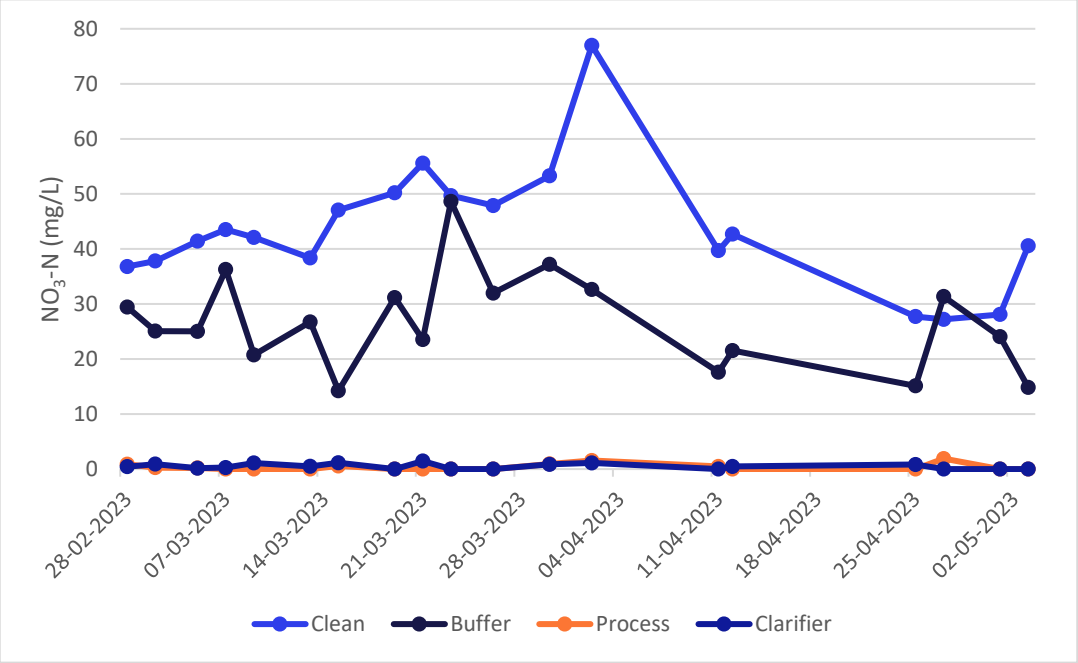

Figure 19. Nitrate concentration in Danish Salmon A/S activated sludge system.

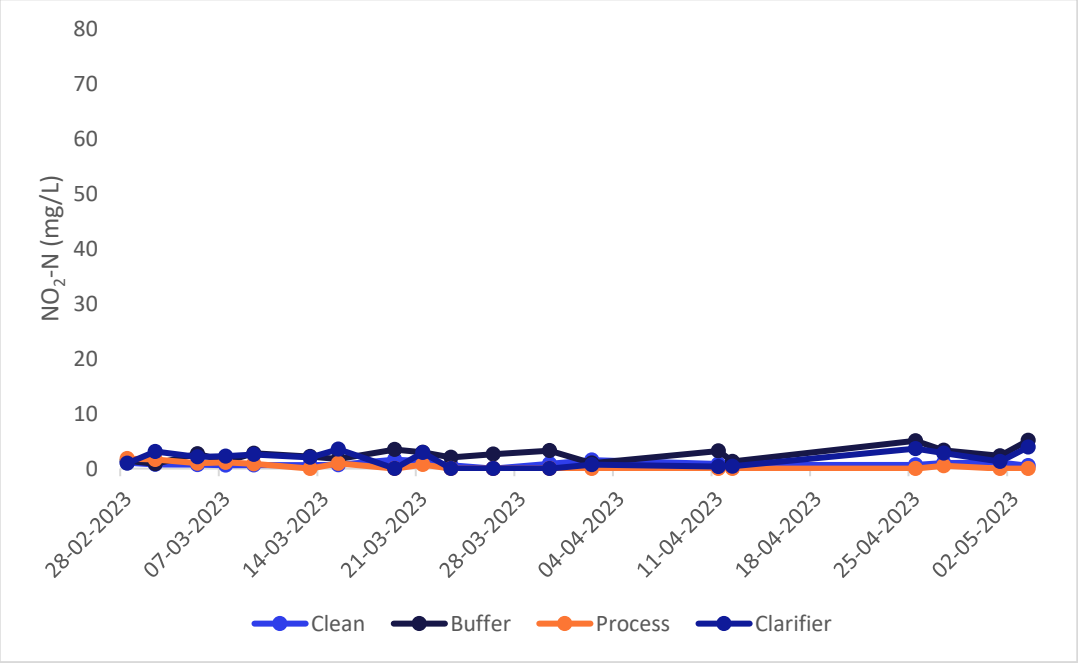

Figure 20. Nitrite concentration in Danish Salmon A/S activated sludge system.

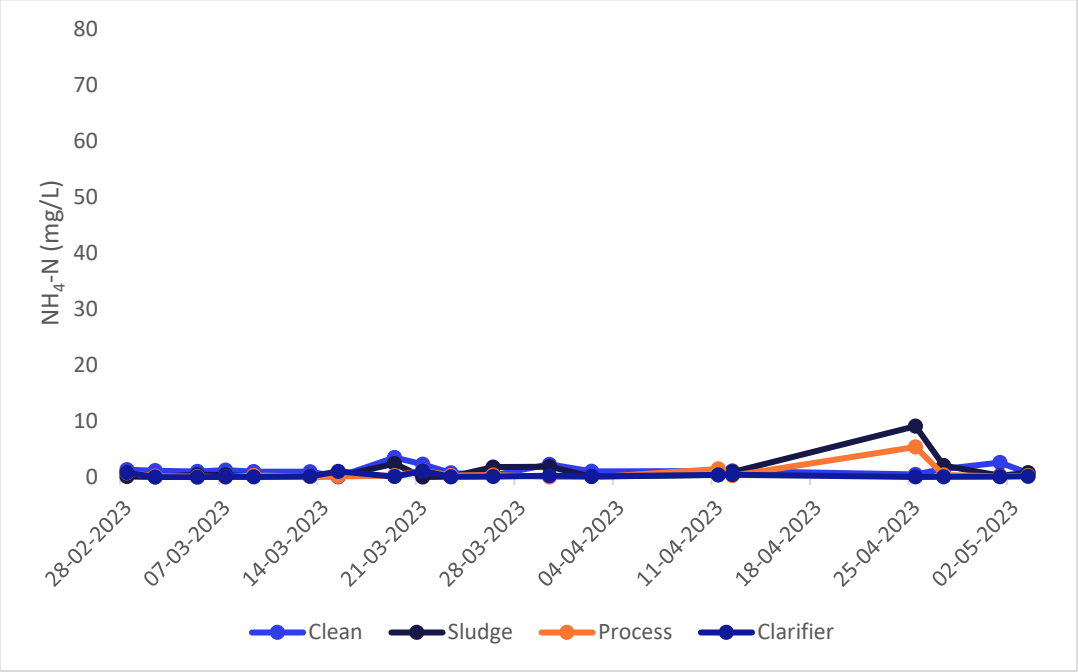

Figure 21. Ammonia concentration in Danish Salmon A/S activated sludge system.

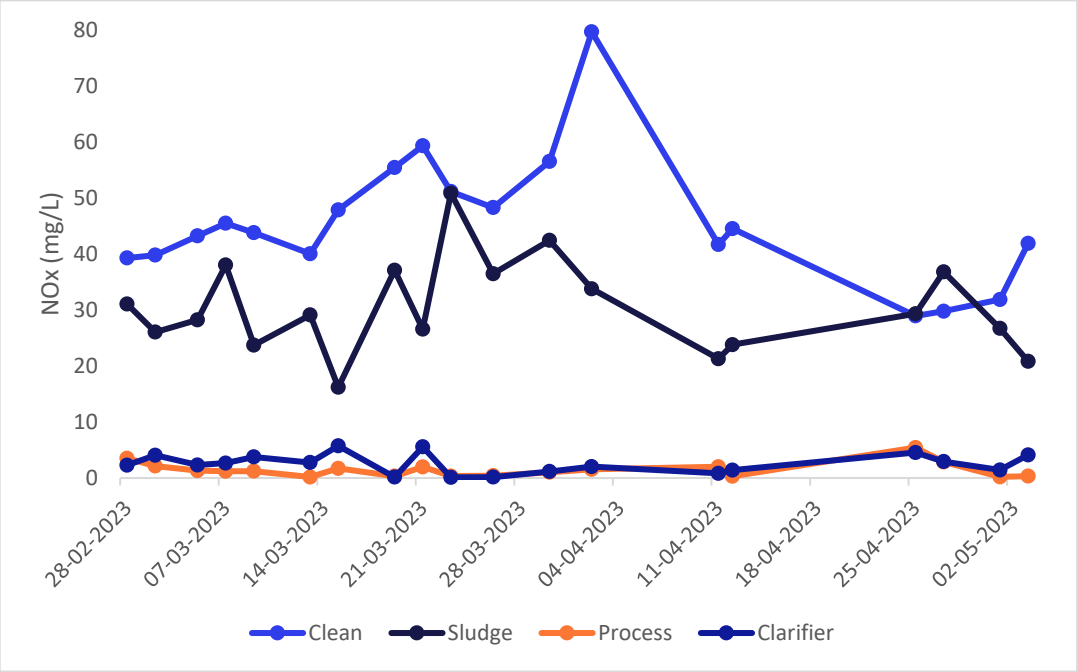

Figure 22. NOx concentration in Danish Salmon A/S activated sludge system.

The calculated daily production of N is 117 kg, of which 75 kg is NH_4^+-N. Given that the total overflow of the farm to the clean tank is 2,394 m^3/ day, the estimated dissolved N (NH_4^+-N, NO_2^--N and NO_3^--N) concentration is 31 mg/ L. The mass calculation shows that the estimated dissolved N produced daily is 116.74 kg/ day. The fluctuations in nitrogenous products across the observation period are reflected in Figure 22.

The modelled daily production of P is 18.7 kg/day of which 6.1 kg/day is PO_4^{3-}-P. The mass calculation indicates that 4.38 kg of PO_4^{3-}-P is delivered to the process tank. The concentration trend of PO_4^{3-}-P is shown in Figure 11.

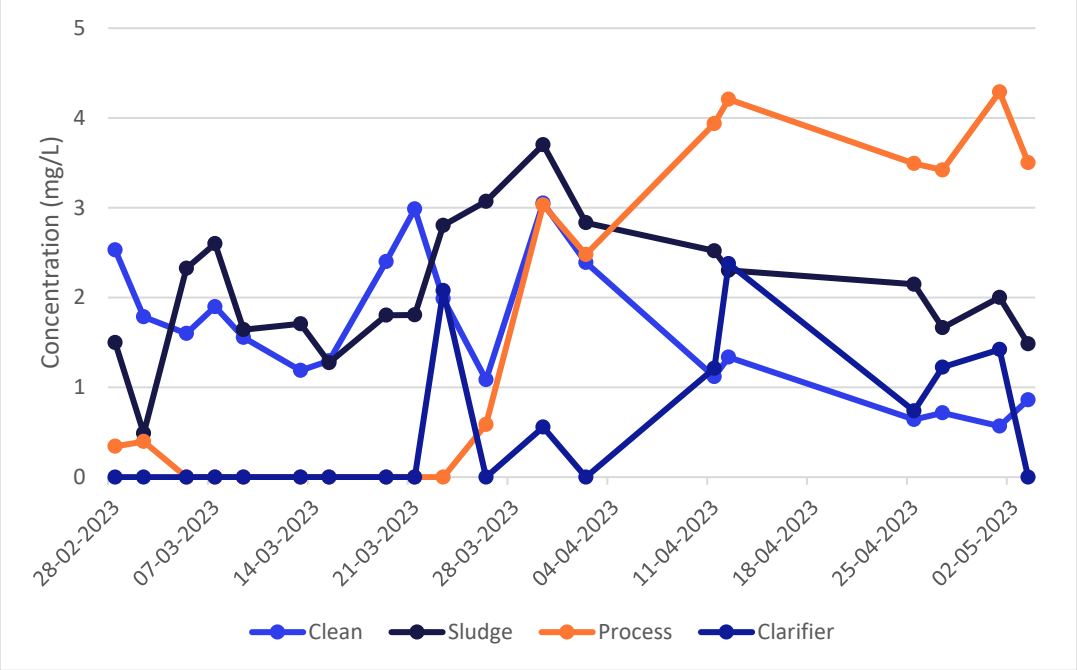

Figure 23. PO_4^{3-}-P concentration in in Danish Salmon A/S activated sludge system.

Based on the mass calculations, the average efficiency of the denitrification process is shown in Table 7.

Table 7. Denitrification treatment efficiency in the volume control, quantity removed by the denitrification processes, corresponding quantity of nutrients discharged into the sea from clean and clarifier tank and the overall treatment efficiency relative to total nutrient production.

Parameter	Efficiency (%)	Quantity removed (kg/day)	Discharged to sea (kg/day)	% of treatment
NH_4^+-N (mg/L)	93.13	0.91	2.62	25.81
NO_2^--N (mg/L)	77.36	0.99	1.86	34.68
NO_3^--N (mg/L)	99.62	26.25	83.41	24.42
Dissolved N	98.20	28.85	87.89	24.71
PO_4^{3-}-P (mg/L)	92.26	1.26	3.20	28.24
TCOD (mg/L)	95.28	478.34	233.51	67.20
sCOD (mg/L)	85.28	52.59	67.59	43.75
Acetate CODeq	96.56	23.35	2.74	39.42*

*This figure discounts the COD equivalent from Acetate added by the farm

The overall removal efficiency of the activated sludge plant at Danish Salmon A/S is overall higher than 93% while smaller efficiency was found for nitrite and ammonia corresponding to a 7% of the total N removed. In an overall overview the treatment capacity of the system

is 24.7% of the total discharge effluents, this means that the farm currently removes approximately 10.5 tons and discharges 32 tons of dissolved nitrogenous products a year. For PO_4-P, the farm treats and removes 0.5 tons and discharges 1.2 tons into the sea per year.

No H_2S was detected in any of the samples but N_2O was detected at varying levels in all 4 tanks (Table 8). The estimated N_2O emissions (emitted from all 4 tanks) is 2,583 g of N_2O/ day which has a CO_2 equivalent of 705 kg CO_2/day. This is equivalent to 1.52 g N_2O/ kg fish or 413 g of CO_2 equivalent/kg fish. The N_2O-N emitted 5.6% of the dissolved nitrogen supplied into the volume control.

Table 8. **Measured N_2O-N concentrations in each tank (average ± SD) and their corresponding estimated emissions.**

Compartment	N_2O-N (g/m3)	Estimated N_2O emissions (g N_2O-N /day)
Clean	0.03 ± 0.15	134.53
Sludge	0.14 ± 0.29	119.03
Process	1.09 ± 1.17	565.29
Clarifier	1.54 ± 1.48	825.00

The solid retention time in the sludge tank is estimated to be 1.3 days. The average SCOD:N ratio which was supplied to the volume control was 2.1 while the TCOD:N ratio was 17. The average HRT in the process tank is 4.5 hours while the HRT in the clarifier is 4.7 hours.

Overall, the denitrification efficiency at Danish Salmon A/S was relatively high. 98% of dissolved nitrogenous products (NH_4^+-N, NO_2^-N, NO_3^--N) that was supplied to the volume control was removed. NO_3-N was the main nitrogenous product entering the process tank (92% of dissolved N), mainly via the clean tank. Both NH_4^+-N and NO_2^--N were present but in relatively low amounts, making up 3% and 5% of dissolved N supplied to volume control respectively. NH_4^+-N was only relatively high in the sludge and process tank on 25 April and a correspondingly high N_2O-N concentration was also measured in the process and clarifier tank. This could be attributed to nitrifier denitrification where ammonia is oxidized to nitrite and subsequently N_2O (Henze et al., 1997a). In wastewater treatment processes, nitrifier denitrification was responsible for most of the N_2O emissions (Li et al., 2008). However, no significant correlation was found between NH_4^+-N and N_2O concentration in the process tank. As such, the N_2O emissions for Danish Salmon likely arises from incomplete denitrification.

Notably, despite the low SCOD/N ratio of only 2.1, the treatment efficiency of SCOD was relatively low at 85%. In addition, the percentage of SCOD to TCOD in the sludge tank is only 3% despite a SRT of 29.5 hours. As such, it is unlikely that more SCOD will be produced in the process tank which only has an HRT of 4.7 hours. This shows that despite operating in a theoretically limited SCOD condition, not all SCOD supplied to the volume control was utilized by the denitrifiers. The low measured production of sCOD can be attained to the fact that the sludge tank utilizes aeration as a mixing device. The oxygen delivered to

the system will force bacteria to oxidize the organic matter with it. Therefore, not all the potential C source are being used and it is a aspect that requires further evaluation and modification if more advantage must be taken form the endogenous carbon sources. Anyhow, the high denitrification efficiency despite the low C/N ratio could be attributed to a mixotrophic denitrification process (Zhang et al., 2023). As the farm uses iron chloride as a coagulant in the sludge tank to assist with the removal of solids via a belt filter, this could promote Fe (II)-based autotrophic denitrification.

The relatively low SCOD efficiency shows that that acetate was not a limiting factor in the denitrification process. In addition, despite the higher acetate concentration observed on 2 April, the N_2O-N concentration in the process and clarifier tank on 2 April was still relatively high. As such, incomplete denitrification and N_2O emissions could be attributed to the effect of oxygen in the denitrification pathway.

While the quantity emitted was only 5% of nitrogenous products, this was equivalent to 0.7 tons of CO_2/day. Given that the price of 1 carbon unit (i.e. 1 tons of CO_2) was approximately 85 euros (Carbon Credits, 2023), the cost of greenhouse gas emissions by Danish Salmon would cost the operations 21,879 Euros or 163,000 DKK annually. Although nitrous oxide emissions are not monitors by the Danish Authorities yet, the significant carbon footprint arising from their denitrification operations needs to be addressed in view of EU's 2030 climate target plan and to become climate neutral by 2050.

The lower nitrate and higher nitrite levels in the sludge tank as compared to the clean tank also shows that denitrification is already occurring there. To facilitate pumps, Danish Salmon aerates the sludge tank to homogenize the sludge. This could explain some of the shortfalls in the modelled carbon output versus the measured carbon output. That the aeration was facilitating the decomposition/ oxidation of the sludge and carbon was escaping as carbon dioxide. The other shortfall in the carbon budget is likely due to the removal of solids via the belt filter.

PO_4^{3-}-P concentrations were relatively stable throughout the observational period in the clean and sludge tanks. However, PO_4^{3-}-P concentrations were rising towards the end of the observational period for both the process and the clarifier tanks. This was preceded by a peak in PO_4^{3-}-P concentrations in the sludge and clean tank. This accumulation of PO_4^{3-}-P could be due to the solubilization of PO_4-P via the hydrolysis and fermentation of the fish sludge (Conroy and Couturier, 2010).

1.13 Results and discussion of UASB

The reactor showed a high volumetric denitrification capacity of 2400 gNO_3-N/m^3 reactor per day, and a specific denitrification rate of 20.3, showing an HRT of 3 h to complete the reduction of nitrate and 6 hours for fully remove nitrite. Even though the denitrification capacity is high it is still low as compared to other studies (Klapwijk et al., 1981; Letelier-Gordo and Herreros, 2019). This difference relays in the fact that the other published results use artificial medium in order to create bacterial biomass, while in this case the fish faeces was the main nutrient source providing micro and micro nutrients.

One of the important factors to have in mind when evaluating denitrification process is the complete reduction of nitrate and nitrite, measured ad total inorganic nitrogen (TIN). In this case nitrate was reduced quite fast (3 hours), while nitrite took 6 hours for complete removal. Important is to see that nitrite levels already were found at the start of the trials, meaning there was previous accumulation of this compound in the process. This can be related to a lack of carbon sources (low C:N) or an incomplete development of the bacterial community in charge of nitrite reduction. Therefore in this case the C:N should have accounted for existing nitrite in the water.

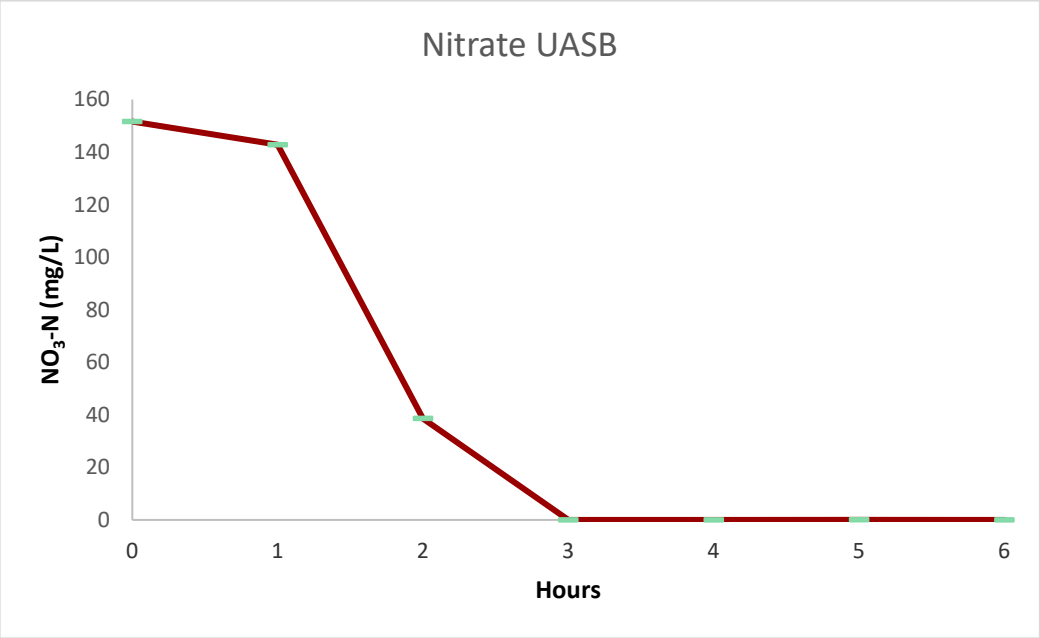

Figure 24. Nitrate removal in UASB reactor.

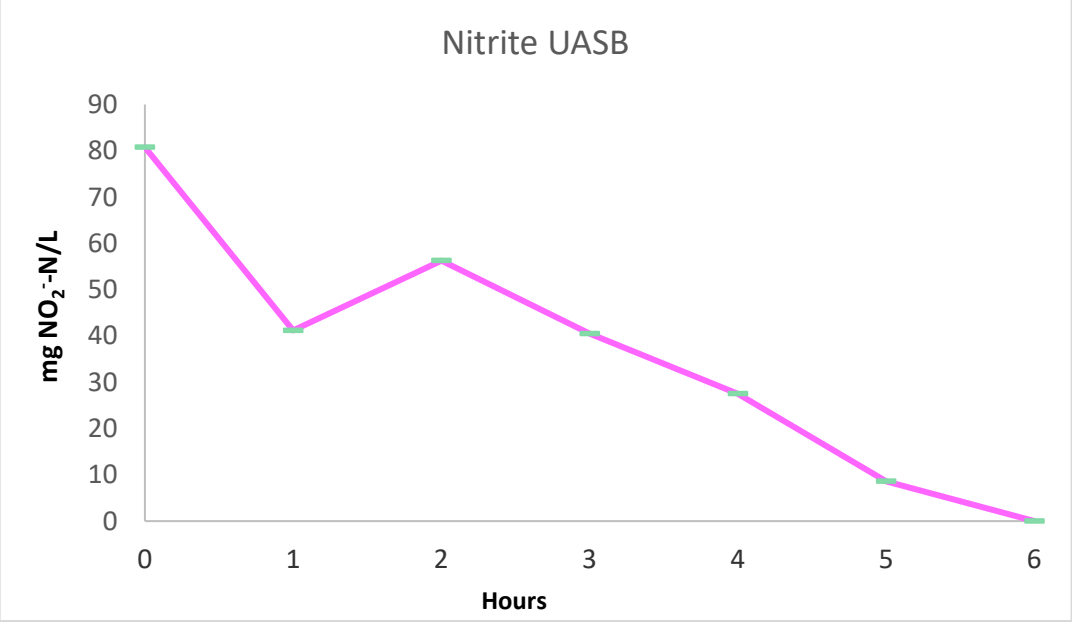

Figure 25. Nitrite removal in UASB reactor.

The results suggest promising technology with high removal capacity and low footprint. One of the problems that required further development is to find a mechanism for which the bubbles entrapped in the flocculant bacteria can be released before the bacteria floats out of the reactor, creating the loss of the biomass. Furthermore, changes in the water quality affect the biomass structure and composition (i.e. sulfide production). This problem occurs when all nitrate is depleted from the reactor and extra carbon sources are available, allowing the production of sulfide (Letelier-Gordo et al., 2020a), which make the decomposition of the bacterial biomass.

1.14 Energetic and resource comparison among N technologies

With the results obtained from the laboratory trials and evaluations done in field a comparison among tested technologies was made. In order to scale and compare energetics demand the following equation was used:

$$W = \frac{Q * Hm * \gamma}{\eta} \qquad \textbf{Equation 12}$$

Where:

W: Consumption (W/h)
Q: Flow (m^3/s)
Hm: Manometric Height (m).
γ: Specific weight of water column (9810 N/m^3)
P: Discharge Pressure
η: Efficiency Equipment

Table 9. Estimated energy consumption for different evaluated technologies.

FBR		**Assumptions**			
N to be treated (kg)	25.9	Q	0.01	m^3/s	
C:N acetate (kg)	155.4	Hm	4	m	
HRT (h)	3.5	γ	9810	N/m^3	
Removal capacity (gN/m^3 reactor*day)	209	Ƞ	65	%	
Vol. reactor m^3	124	W	6	kwh	Discharge pump
Flow (m^3/h)	35.3		6	kwh	Input pump
Energy (kw/kg N)	2.7		2	kwh	Mixer
		Energy consumption	14	kwh	

Activated sludge		**Assumptions**			
N to be treated (kg)	25.9	Q	0.02	m^3/s	
C:N acetate (kg)	155.4	Hm	4	m	
HRT (h)	4.5	Γ	9810	N/m^3	
Removal capacity (gN/m^3 reactor*day)	82	Ƞ	65	%	
Vol. reactor m^3	316	W	12	kwh	CSTR pump
Flow (m^3/h)	70.2		2	kwh	Mixer
Energy (kw/kg N)	12.8				
		Energy consumption	14	kwh	

UASB		**Assumptions**			
N to be treated (kg)	25.9	Q	0.02	m^3/s	
C:N acetate (kg)	155.4	Hm	5	m	
HRT (h)	4.5	γ	9810	N/m^3	
Removal capacity (gN/m^3 reactor*day)	2400	Ƞ	65	%	
Vol. reactor (m^3)	11	W	2	kwh	In pump
Flow (m^3/h)	2		2	kwh	Mixer
Energy (kw/kg N)	3.9				
		Energy consumption	4	kwh	

1.15 Summary and future development on N removal technologies

In Denmark, the production capacity of aquaculture farms is limited by their nutrient discharges. As such, the more effluent the farms can treat, the more they are allowed to produce. Thus, this work on denitrification would allow for decoupling of aquaculture production from environmental pressure in terms of nutrient discharges.

Currently legislative requirements only apply to nitrate and nitrite discharges. However, in view of the ongoing climate change crisis, nitrous oxide emissions will be more relevant and important in the coming years. As such, it is important to understand ways to limit the production of nitrous oxide. Thus, this research on C/N ratios and hydraulic retention time contributes to the understanding of factors to ensure complete denitrification. In addition, by assessing the current effectiveness of denitrification at a commercial facility, we also now understand the amount of nitrous oxide that could be produced from an aquaculture facility.

This research has shown that the elimination of nitrate and nitrite can be done relatively effectively with sufficient denitrifying bacteria. However, eliminating the production of nitrous oxide requires further research. A potential research direction could be to utilize autotrophic denitrification instead of heterotrophic denitrification which has lower CO2 emissions.

Experimental methodology for the C/N batch trial and the Feed Batch Reactor can also be further developed to expand on this research topic.

The C/N ratio experiment can be repeated with a higher concentration of denitrifying bacteria or bacteria which are more mature to observe the 2nd/ 3rd/ 4th step reaction in the denitrifying pathway. Measurements can also be done to determine the percentage of N_2O-N emitted in relation to each C/N ratio when all nitrate and nitrite is reduced.

The optimization of FBR experiment can be done by decreasing the HRT with the correct SCOD:N ratio supplied. As the FBR design has proven to conduct denitrification successfully with a quick set up time, further investigations can be made such as the alteration of the C/N ratio and analysis of the bacterial biome in the reactor. N2O measurements can also be made to establish a better understanding between C/N ratios, HRT and N2O emissions.

For Danish Salmon, further investigations can be made to confirm if mixotrophic denitrification was occurring.

2. Organic matter and phosphorous removal

2.1 Coagulation and flocculation

Coagulation and flocculation are the most common processes for the removal of dissolved P and suspended organic matter in RAS. These two processes are usually combined in a two-step configuration where coagulation is done through the application of hydrolyzing metal salts (i.e.$AlSO_4$ and $Fe^{3+}CL$) or already hydrolyzed metals as polyaluminium chloride (PAC) followed by the application of organic polymers (flocculants) with high molecular weight (i.e. Polyacrylamide, polydiallyldimethylammonium).

In the coagulation step, three main processes can occur; 1) Destabilization of small suspended and colloidal particulate matter 2) Adsorption or reaction among the colloidal and dissolved organic matter into particles 3) Creation of flocculant particles that will sweep through the water for posterior treatment. The coagulants such as alum or ferric salts will hydrolyze in water to form insoluble precipitates that will destabilize particles through neutralizing charges or surface adsorption, reducing repulsive forces and thus creating particle agglomeration.

2.1.1 Mechanism behind the process

The main mechanism behind removal of suspended solids consists in the reduction or neutralization of the ζ-potential (electrical double layer), promoting the interaction between particles to form larger aggregates defined as flocs (Letterman et al., 1999a). The ζ-potential, or electrical double layer, is a cloud of ions surrounding the particle that satisfy electro neutrality and is the main reason why these particles remain in suspension for long periods of time without aggregating (Weiner and Matthews, 2003). From the many factors that affect double-layer thickness, ionic strength is perhaps the most important (Letterman et al., 1999b). A compressed double layer will result in particles bonding due to Brownian motion and remaining attached due to van der Waals forces of attraction (Crittenden et al., 2012; Henze et al., 1997b). As the ionic strength of a solution is increased, the extent of the double layer decreases, which in turn reduces the zeta potential. For example, in seawater, the ζ-potential is 0.70 M, and in river water 0.0017 M (Nazaroff & Cohen, 2001). Coagulation applied on desalinization processes have proven that in salt water the higher ionic strength (cations and anions) facilitates the general coagulation process, making it more efficient as compared to coagulation in freshwater (Duan et al., 2002).

The purpose of flocculation is to enhance the coagulation process by promoting the aggregation and floc formation that can efficiently be removed in subsequent separation processes such as sedimentation, flotation and coarse filtration. Normally a flocculant is added after coagulation process once the particle or ion is destabilized or adsorbed. A series of mechanisms are behind the flocculation process, as Brownian motion, velocity gradient in laminar flows, inequal settling velocities and turbulent diffusion. These mechanisms follow a second order rate process in which the rate of collision between the target particle and the flocculant is proportional to the product of concentrations if the two colliding units.

2.1.2 Classification of coagulant and flocculants

Coagulants/flocculants can be classified in two main groups, chemical and natural. Chemicals can be subdivided into; hydrolyzing metal salts (e.g., alum sulfate and ferric chloride), inorganic polymers (poly aluminum chloride (PAC), and synthetic organic polymers (polyethyleneimine and polyacrylamide (PAM), while natural can be plant-based (tannin), animal based (chitosan) and microorganisms-based (fungi).

2.1.3 Process dependent parameters

The coagulation/flocculation process depends largely on the type of selected chemical to be used. The array of chemicals available for this process is immense and the proper selection will depend on the different structural characteristics as ionic properties, functional groups, molecular weight and the type of water to be treated due to its ionic strength (zeta potential) found from saline to saltwater. Because most of the suspended particles in water has a negative surface charge, coagulants normally contain trivalent metal inorganic salts (aluminum sulfate, ferric chloride, polyaluminium chloride) in order to obtain a high charge neutralization. On the other hand, flocculants being mostly organic polymeric materials their effect rely on the high molecular weight to enhance bridging flocculation.

Apart from the intrinsic coagulant/flocculant structural properties and charge, the most important factors that affect its use and removal capacity are, dosing, ionic strength, pH, temperature and mixing conditions. Underdosing of coagulant/flocculant leads to low removal of P and organic matter, while an overdose results in an excess of chemical sludge (Letelier-Gordo and Fernandes, 2020). The pH of the treated water will affect the surface charges of colloidal particles which will affect the charge neutralization effect. Temperature affects the viscosity of water as higher viscosity (low temperature) has a negative effect on Brownian motion and decreased agglomeration. The type of mixing condition will affect the efficiency of the process. Normally rapid mixing is followed by slow mixing. Rapid mixing (250 rpm) occurs right after coagulant dosage increasing the chances of contact between particles and the coagulant, while slow mixing (30 rpm) will enhance the aggregation of flocs.

2.1.4 Evaluation method

Jar tests are widely used for evaluating the efficiency of coagulant and flocculant against a target parameter. It is a laboratory test that simulates the full-scale coagulation/flocculation process and can be conducted in a wide range of conditions. The setup consists in a series of parallel stirrers with variable speed capacity. During the test 1 or more liters of water are placed in each container and different coagulant/flocculant doses are applied. Firstly, a rapid mixing (250 rpm) for 5 min followed by a slow mixing (30-50 rpm) for 15 min. Samples for P and TSS are taken before and after treatment at 80% of the container high including the control (no chemical). Sludge volume index or the heigh of the sludge after treatment is also measured to evaluate the volume of the treated sludge.

2.2 Research aims for coagulation and flocculation

The aim objectives of this part of the study were to:

- Evaluate the efficiency of different coagulant performance in fresh and saltwater conditions for removing P and TSS
- Create a dose response evaluation of the most efficient ones

- Evaluate the effect of combined coagulation-flocculation
- Develop a pilot scale P and TSS removal device

2.3 Materials and methods

Coagulant-flocculant stock solutions and sludge samples preparation
Different doses of various chemical coagulants (AluPAC, AluSAL, and AluACH), organic coagulants (BoGreen 1, 2, and 6), and blend coagulants (AluBlend 610A, 610B, and 710A) were systematically evaluated (table 10). In addition, some coagulants were tested with BoGreen 5 and BOFLOC (anionic) flocculants. The coagulants and flocculants were developed and produced by Alumichem Inc., Denmark.

For preparing the coagulant/flocculant stock solution a certain volume of coagulant-flocculant was extracted from the respective bottle using a pipette. The volume previously weighted using a microscale (Metler Toledo, XP204) was poured and diluted into a 200 mL volumetric flask. The content was mixed at 250 rpm for 30 minutes using a magnetic stirrer (Big Squid, IKA, Germany).

After 24 hours of fish feeding, 1L of fish sludge sample was taken from the sludge collectors adjacent to the fish tanks. The sample was poured into 2L methacrylate beakers (Duran@, Denmark).

Modified jar test description
To evaluate the removal efficiency of reactive phosphorous (PO_4^{-3}), turbidity and the production of chemical sludge, measured as settling sludge volume final (SSVf), a modification of the jar test (Lee and Lin, 2000) was performed. The procedure was conducted in 1L graduated tubes (Duran@, Germany) and magnetic stirrers (Big Squid, IKA, Germany). Different volumes of the coagulants and flocculants stock solutions were added to the sludge sample, and a positive control (no coagulant, only stirring) were evaluated in replicates (n = 3).

The screening experiment was performed by adding 6 mL of coagulant stock solution to the sludge sample, the temperature remained constant at around 18 ± 0,5 ºC (room temperature), and the salinity tested was 0 (freshwater) and 28 (saltwater) ppm. The Dose-response experiment was performed by adding 6, 9 and 12 mL of coagulant stock solution into the sample, the coagulants tested were AluPAC, AluSAL, AluACH, BoGreen1, Alublend610A, and AluBlend710A.

Finally, the coagulant-flocculant experiment was performed by adding 14.4 mg of BoGreen 5 and 9,5 mg of BOFLOC in addition to the coagulants which had the best performance during the dose-response experiment (12 mL of AluSAL and AluACH). Dose-response and coagulant-flocculant experiments were performed at 18 ± 0,5 ºC and 28 ppm of salinity (Table 10).

Table 10. **Experimental design. Types and volumes of coagulant stock solutions, temperature, and salinities of tested treatments. Amounts of flocculants tested in the coagulant-flocculant experiment. The experiments were carried out using 1L fish sludge.**

Type of coagulant	Coagulant	Flocculant	Volume coagulant stock solution	Temperature	Salinity
Screening					
Chemical	AluPAC AluSAL AluACH	X	6 mL	18 ± 0,5 ºC	0 ppm 28 ppm
Organic	BoGreen 1 BoGreen 2 BoGreen 6	X			
Blend	AluBlend 610A AluBlend 610B AluBlend 710A	X			
Dose-response					
Chemical	AluPAC AluSAL AluACH	X	6 mL 9 mL 12 mL	18 ± 0,5 ºC	28 ppm
Organic	BoGreen 1	X			
Blend	AluBlend 610A AluBlend 710A	X			
Coagulant-flocculant					
Chemical	AluSAL	14,4 mg BoGreen 5 9,5 mg BoFloc	12 mL	18 ± 0,5 ºC	28 ppm
	AluACH	14,4 mg BoGreen 5 9,5 mg BoFloc	12 mL	18 ± 0,5 ºC	28 ppm

The jar test protocol consists of three phases: 1) a flash mix, where the coagulant-flocculant and the solution are mixed at high rotation (5 min at 500 rpm); 2) followed by a slow mix at a lower speed (25 min at 250 rpm); 3) and finally a quiescent settling, where the samples are transferred to 1 L graduated cylinders and allowed to settle (30 min).

Figure 26. Coagulation and flocculation jar test screening trials using different chemicals.

Figure 27. Physical appearance of coagulated and flocculated sludge.

pH measurements were taken before and after the modified jar test procedure. Water samples were collected at the 80 % upper height from the respective jar test tube and at the end of the quiescent settling period (1 h after coagulant addition).

2.3.1 Analytical methods

The samples for reactive phosphorous, expressed as $PO_4^{-3} - P$, were filtered through 0.2-µm syringe filters (Filtropour S, SARSTEDT, Germany) and analysed using Ion Chromatography (930 Compact IC Flex 1 with a Metrosep A Supp 7 -250/4.0 column, combined with an 887 Professional UV/VIS detector; Metrohm, Sweden) using 0.1 M H2SO4 as suppressor and 3.6 mM Na_2CO_3 as eluent. Turbidity was measured in formazin nephelometric unit (FNU) using EQUIPMENT and final settled sludge volume (SSVf) was determined according to 2710D (Eaton et al., 1995).

2.3.2 Data treatment

Reactive phosphorous and turbidity removal efficiencies of the samples obtained from the Jat test, were calculated following (Eq.13).

$$\boldsymbol{\eta}\ (\%) = \frac{\boldsymbol{Ci - Cf}}{\boldsymbol{Ci}} * \boldsymbol{100} \qquad \text{(Equation 13)}$$

Where:
Ci: concentration of the parameter tested in raw samples
Cf: concentration of the parameter after the jar test procedure.
When η is positive it means the removal of substance while a negative value means accumulation.

2.3.3 Statistical analysis

Differences in the removal efficiencies for $PO_4^{-3} - P$, TSS, and settled sludge volume final (SSVf) between different coagulant-flocculant dosing and control were examined by one-way ANOVA analysis, followed by Tukey–Kramer multiple comparison of means test, with a 95 % family-wise confidence level. Differences were considered significant when P < 0.05, and values are stated as the mean ± standard deviation (SD). The statistical analyses were carried out using the R software (R CoreTeam, 2016).

2.4 Results

2.4.1 Screening

Chemical coagulants based on aluminium had the highest removal performance among all coagulants. After the same dosage of coagulants, the results showed that AluSAL can reach an orthophosphate removal of 70% in seawater, whereas these values for AluPAC and AluACH were 37% and 25% respectively. Between organic coagulants, AluBlend 610A and 610B had a PO_4^{3-}-P removal rate of 35% in seawater, and Alublend 710A had a lower removal rate (25%). All the blend coagulants had removed 90% of the turbidity in high salinity (SW).

Organic coagulants didn't have any phosphorous removal differences compared to the control treatment. However, BoGreen 1 and 2 performed better for turbidity removal in freshwater (85%) whereas BoGreen 1 and 6 had a higher turbidity removal in seawater (87%). Chemical and blend coagulants had a turbidity removal efficiency between 92 and 97% in salt water.

Generally, all the coagulants performed better in salt water than in freshwater, excluding BoGreen 2.

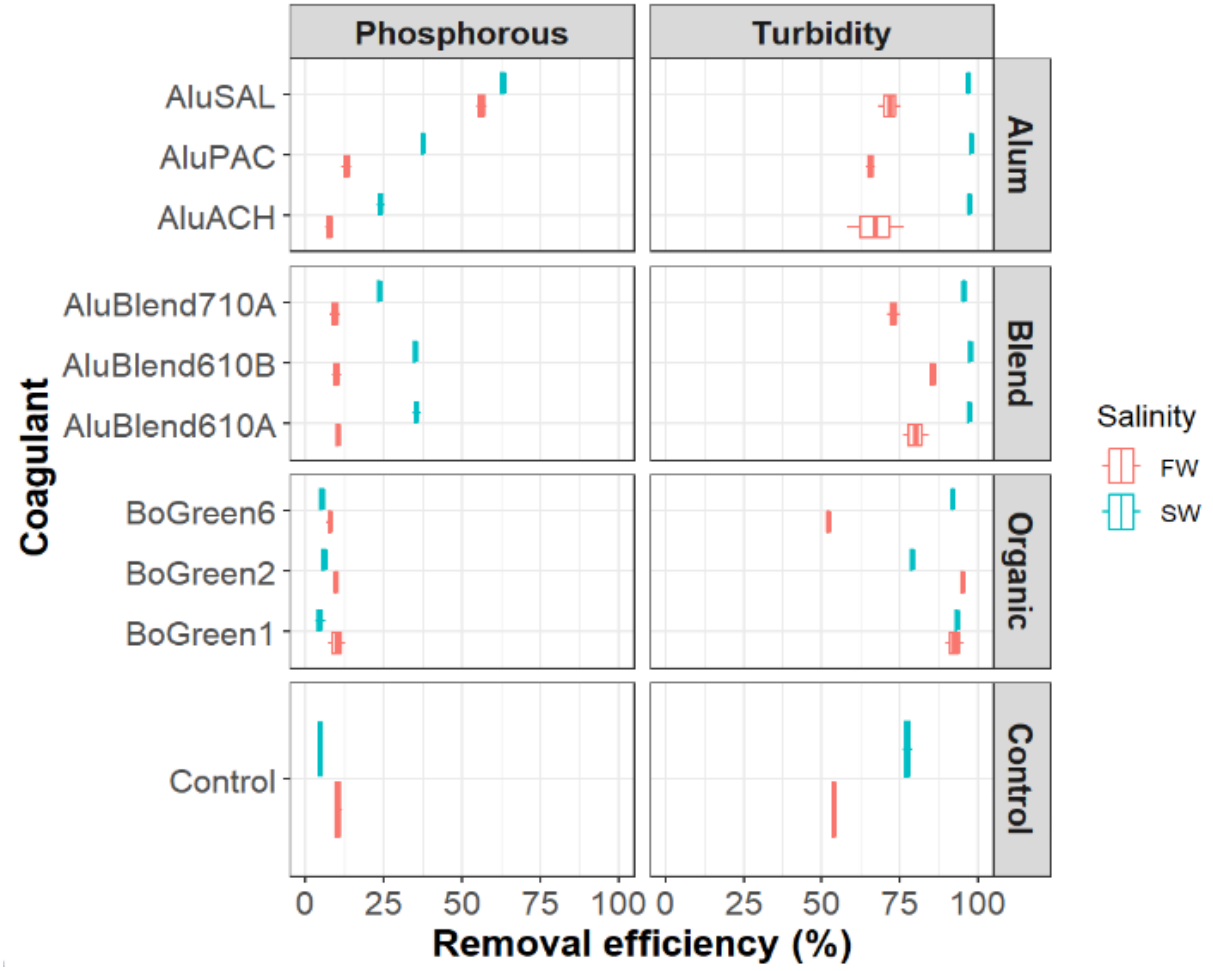

Figure 28. Screening of different coagulant and flocculant using a modfied jar test method from salt (SW) and fresh water (FW) RAS.

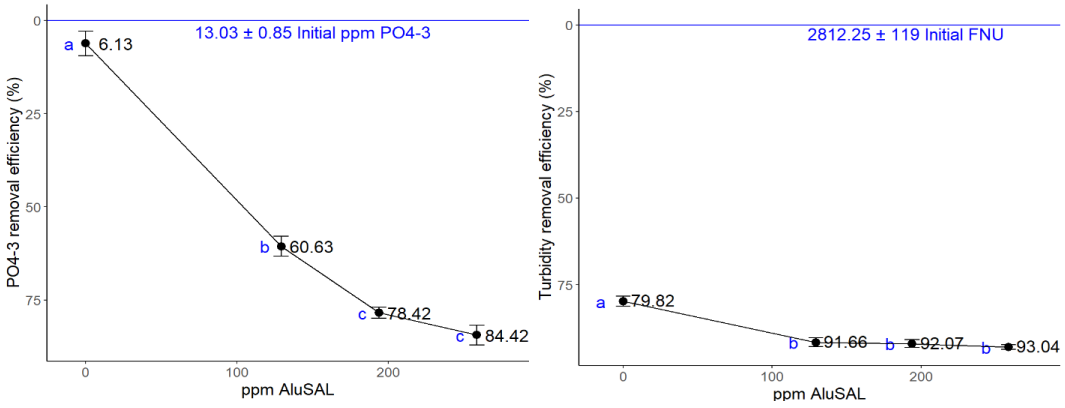

Figure 29. Phosphorous and turbidity removal using AluSal at different dosages in water.

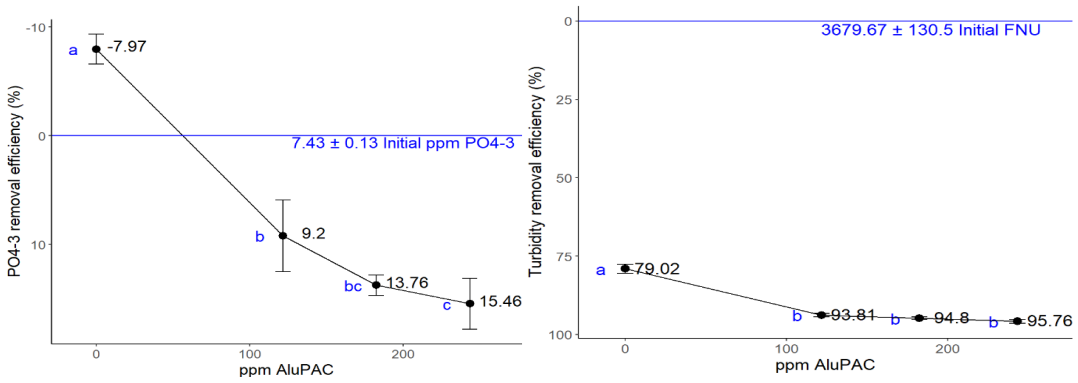

Figure 30. Phosphorous and turbidity removal using AluPAC at different dosages in water.

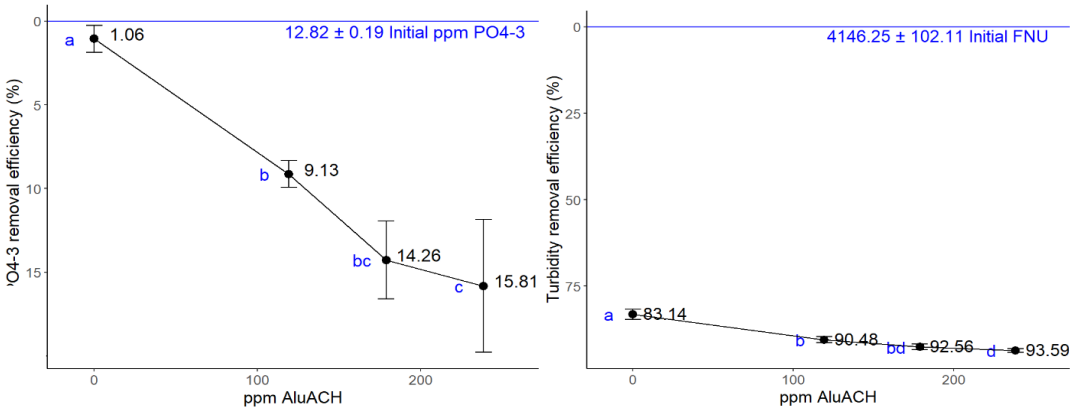

Figure 31. Phosphorous and turbidity removal using AluAch at different dosages in water.

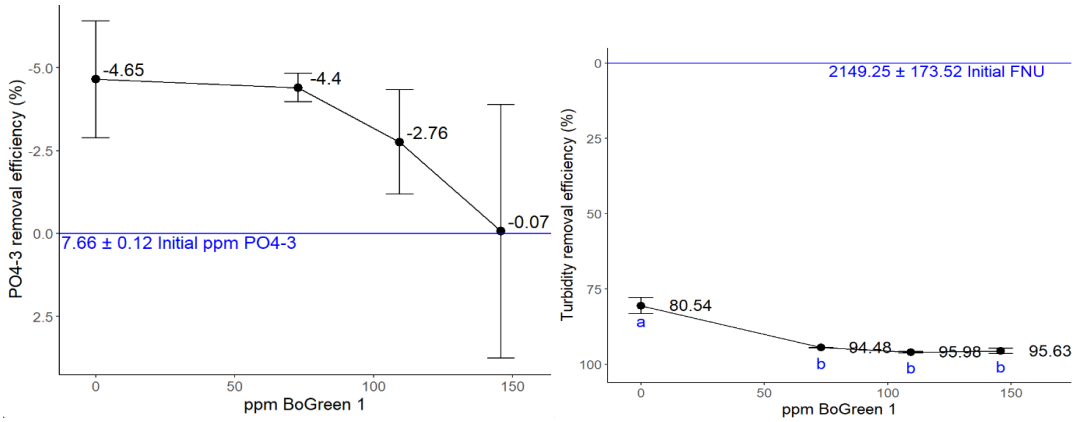

Figure 32. Phosphorous and turbidity removal using BoGreen 1 at different dosages in water.

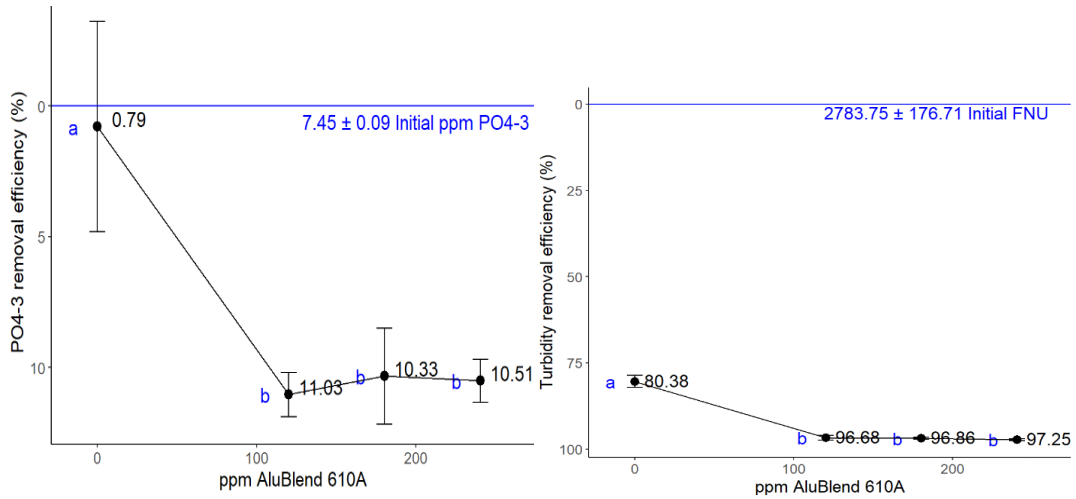

Figure 33. Phosphorous and turbidity removal using Alublend 610A at different dosages in water.

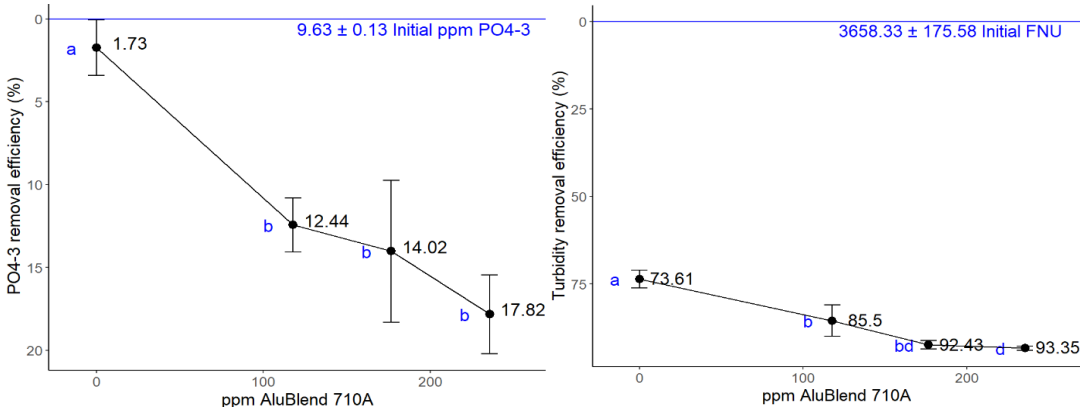

Figure 34. Phosphorous and turbidity removal using AluBlend710A at different dosages in water.

Starting with an initial orthophosphate concentration of 13.03±0.85 mg/L, the removal efficiency increases as the AluSAL coagulant concentration increases. At 0 ppm AluSAL (Control), the removal efficiency is minimal at 6.13%, significantly rising to 60.63%at 129 ppm, then to 78.42% at 194 mg/L, and finally reaching 84.42% at 258 mg/L.

AluPAC and AluACH follow the same trend with significant differences, Fig 29 and Fig 30 Nevertheless, these coagulants reach a maximum orthophosphate removal of 15% around 240 mg/L of coagulant. Figure 32 represents an initial orthophosphate concentration of 7.66±0.12 mg/L, as indicated by the horizontal blue line. The data indicates a positive trend between the concentration of BoGreen 1 and PO_4^{-3} removal efficiency with no significant differences. At 0 mg/L of BoGreen 1, the removal efficiency is −4.65%, indicating a slight increase in phosphate concentration rather than removal. As the concentration of BoGreen 1 is raised to 73 mg/L, the efficiency slightly improves to −4.4%, then to −2.76% at 110 mg/L, and finally to −0.07% at 146 mg/L.

Finally, the blend coagulants (Fig 33 and 34), showed a significant difference between control and coagulant addition treatments. Alublend 610A reached a PO_4^{-3} removal efficiency of 10.51 % at 240 mg/L coagulant, whereas AluBlend 710A reaches a value of 17.82 % of PO_4^{3-}-P removal at 235 mg/L coagulant.

All the coagulants showed significant differences in turbidity removal efficiencies between control and coagulant-added treatments. The highest turbidity removal (97.25%) is reached by AluBlend 610A at 240 mg/L. However, all coagulants achieve turbidity removal around 93-97 %. The control groups obtained a turbidity removal of around 80% excluding the trial with AluBlend 710A.

Table 11. Alu series coagulants doses applied in the evaluation.

Coagulant	**AluSAL**				**AluPAC**				**AluACH**			
Treat. (ppm)	0	129	194	259	0	122	182	243	0	119	179	238
SSVf (mL)	79.82	91.66	92.07	93.04	79.02	93.81	94.8	95.76	83.14	90.48	92.56	93.59
Δ pH	0.16	0.26	0.47	0.59	-0.3	-0.41	-0.46	-0.56	-0.11	-0.18	-0.19	-0.21

Table 12. Blend series applied in the evaluation.

Coagulant	**BoGreen1**				**AluBlend 610A**				**AluBlend 710A**			
Treat. (ppm)	0	73	109	146	0	120	180	240	0	118	177	236
SSVf (mL)	80.54	94.48	95.98	95.63	80.38	96.68	96.86	97.25	73.61	85.50	92.43	93.35
Δ pH	-0.25	-0.22	-0.26	-0.25	-0.26	-0.42	-0.50	-0.58	-0.11	-0.07	-0.09	-0.13

Figures 35 a and b indicates that the addition of flocculants after AluSAL doesn't affect significantly the PO_4^{-3} removed, however, the turbidity removal is significantly improved by adding flocculants. The addition of BoFloc and BoGreen 5 after AluSAL increases slightly the turbidity removal.

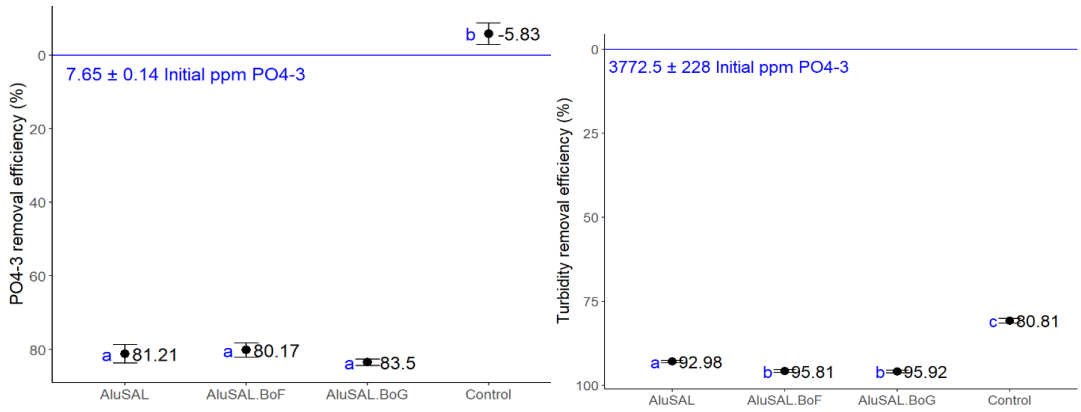

Figure 35a and b. Effect of flocculant dosage on removal of PO_4^{3-}-P .

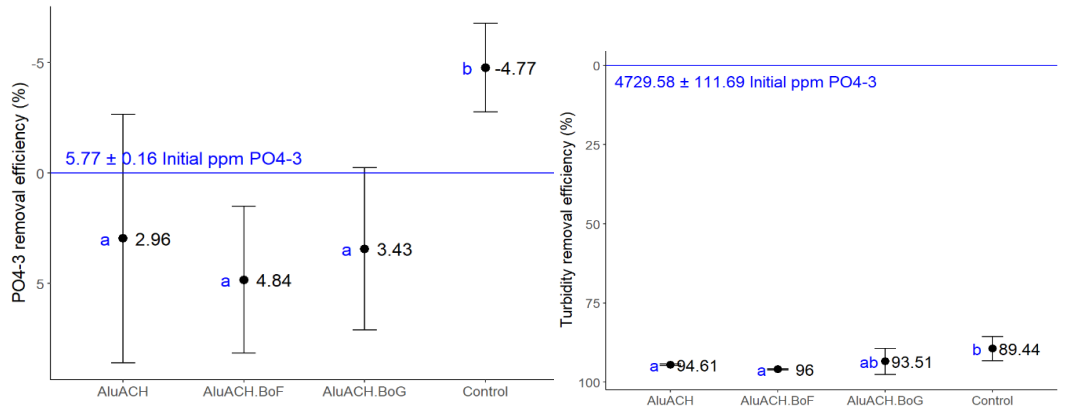

Figure 36. Effect of flocculant dosage on removal of PO_4^{3-}-P.

Figures 36 a and b, indicate the interaction of AluACH and flocculants on orthophosphate and turbidity removal. Both indicate a significant difference between the control and test treatments, the addition of flocculant does not affect significantly the PO_4^{-3} removal efficiency of AluACH. Adding BoFloc or BoGreen 5 to AluACHT does not affect the turbidity removal of AluACH significantly.

Table 13. Flocculant applied after coagulation process

	AluSAL				**AluACH**			
Treat. (ppm)	Control	AluSAL	AluSAL + BoF	AluSAL + BoG	Control	AluACH	AluACH + BoF	AluACH + BoG
SSVf (mL)	196.67	280	216.67	273.33	73.61	85.50	92.43	93.35
Δ pH	-0.17	0.09	0.07	0.10	-0.22	-0.29	-0.29	-0.13

2.5 Description of phosphorus removal plant

The treatment system is designed to meet the specified requirement of less than 50 mg/L Total Suspended Solids (TSS) and 0.5 mg/L of Total Phosphorus (TP) in the treated water

effluent at a maximum flow rate of 110 m^3/h – phosphorus infeed effluent water stream must not exceed 25 mg/L.

2.5.1 Capital expenditure (CapEx)

Breakdown of one-time costs associated with acquiring the technology (hardware, software, installation and setup costs) (Table 14)

Table 14. Cost description of items required for P removal plant.

Cost description	Cost (EUR)
Hardware	
Feed pumps	13400
Flowmeter	1100
Chemical dosing pumps	3500
pH transmitter	1000
Retention tank	30000
Mixer for retention tank	18100
Polymer makeup unit with polymer dosing pump	5700
Drum filter	40000
Control cabinet	24120
Phosphorous sensor	17600
Piping & valves and installation	13400
Installation	20100
Programming	16080
Total	**204100**

2.5.2 Operating expenditure (OpEx)

Breakdown of ongoing operational costs incurred to maintain and use the technology are described in Table 15

Table 15. Cost description of P removal technology.

Cost description	Cost (EUR/Yr.)
Coagulant	*
Polymer solution	*
Personnel costs for training, operation, and support	4000
Utility costs (e.g., electricity, internet)	*
Maintenance and repair costs	3000
Insurance costs	Not applicable
Total	**7000[a]**

*Chemical and utility costs are capacity dependent. Assuming a 100 ml/m^3 of coagulant and 8 l/m^3 for a 4ppm polymer solution, power consumption would approximately be 5-20 kW/m^3 treated. Price of coagulant as of June 2024 is 512 EUR/MT and price of polymer as of June 2024 is 3360 EUR/MT.

[a] Total cost excludes utility and chemistry costs.

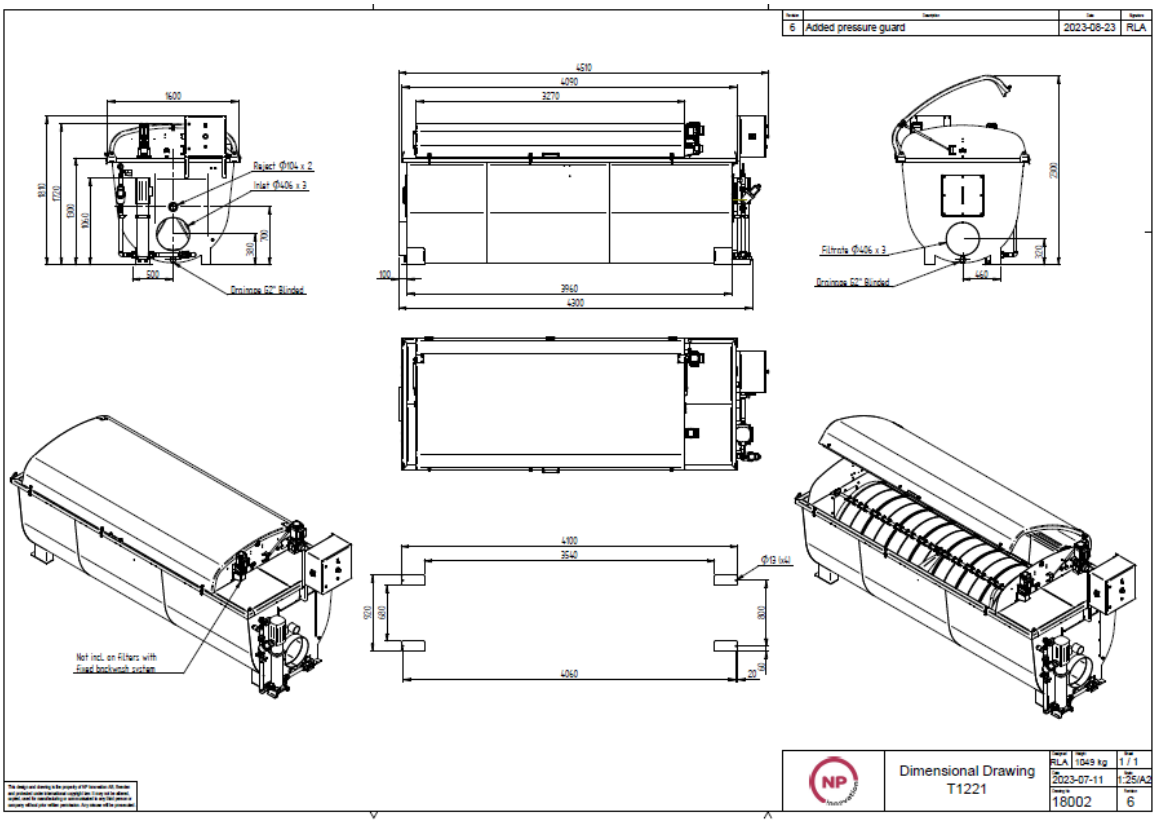

Figure 37. Blueprint of P removal technology.

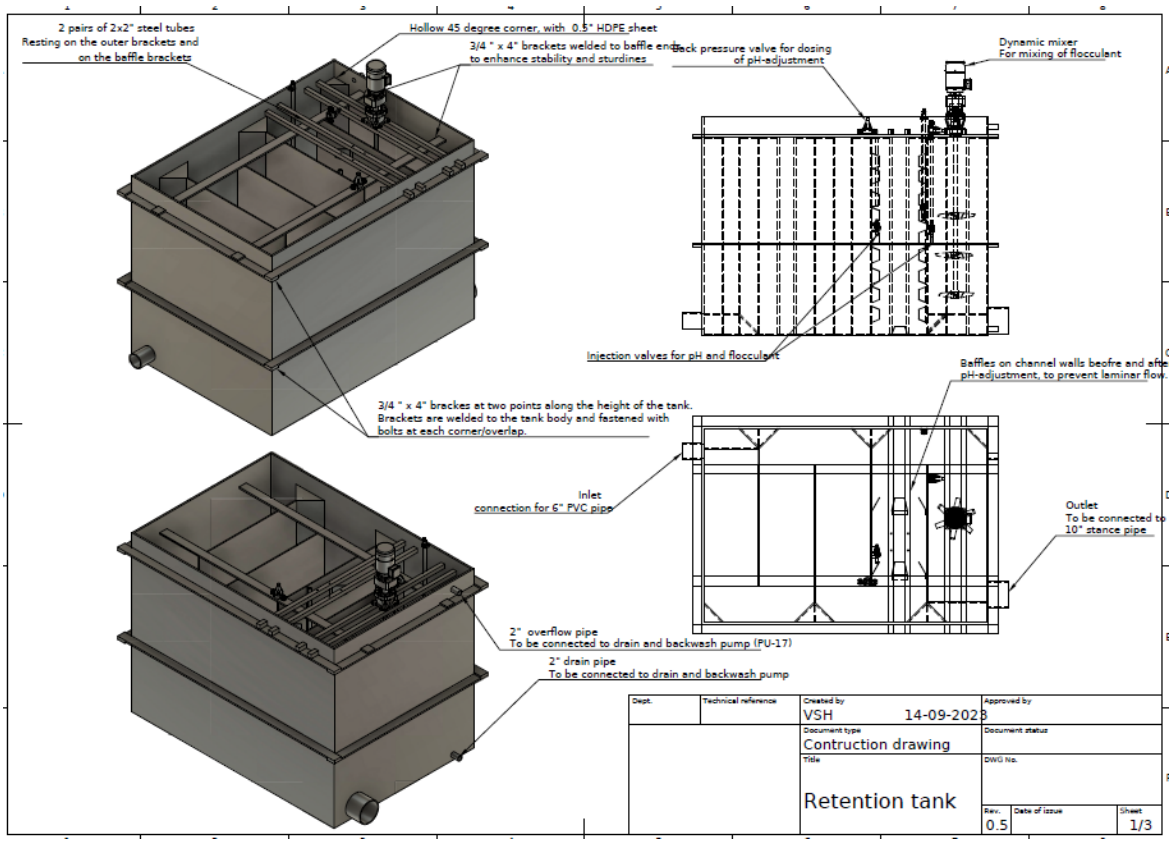

Figure 38. Blueprint of P removal technology.

2.6 Summary and future development on P and suspended solids technologies

The big array of coagulant and flocculants showed different removal capacities for P and suspended solids removal. The effect on the coagulation and flocculation process is affected by salinity, showing higher removal capacities when saltwater was treated. This responds to the fact that saltwater has a higher ionic strength and thus reducing the Z potential and thus promoting coagulation and flocculation mechanisms. The best results for P removal were obtained with the inorganic coagulants while organic based coagulants show a minimum effect on P removal but a higher removal in suspended solids.

The fact that organic coagulants don't remove phosphorous, but they do remove suspended solids is not a negative situation as now a days and with a circular economy strategy there is a need of products that can remove particles from water without affecting the P levels which are required in the case of low trophic cultivation (i.e., seaweed, microalgae and aquaponics).

3. Valorisation of fish manure

Fish manure has a potential high nutrient content creating the opportunity to achieve nutrient circularity by recycling fish waste products into agriculture commodities. Dried fish manure was calculated to have a relative agronomic efficiency of 50-80% compared with mineral fertilizer (Brod et al., 2017). In addition, N and P fertilizer effects were also found in salmon and catfish manure on ryegrass (Lolium multiflorum L.) and lettuce respectively. However, fish manure has a high-water content (1% dry matter), increasing transportation costs and reducing its suitability for application as soil enhancer. Furthermore, fish manure contains micronutrients as well as heavy metals as Cd and Ni which their accumulation in soil or water reservoirs is of major concern since they are not biodegradable and possess risk for human health (Miljøstyrelsen, 1994). Therefore, increasing fish manure dry matter and reducing heavy metal content are aspects that needs to be addressed if fish manure is meant to be used as fertilizer or soil improver.

The European Commission had set a target of having at least 25% of EU's agriculture land to be used for organic farming and significantly increase organic aquaculture by 2030 under the European Green Deal Farm to Fork strategy In line with the objectives of the European Green Deal and the Circular Economy Package, Regulation (EU) 2019/1009 allows for more organic and waste-based fertilizers to be freely traded within the EU (*European Commission-Fact Sheet Circular economy: New Regulation to boost the use of organic and waste-based fertilisers*, 2016). By increasing market access for such fertilizers, it encourages bio-waste recycling derived from waste organic materials. This reduces EU's dependency on raw material imports by recycling existing waste resources. It was calculated that recycled bio-waste fertilizers could substitute up to 30% of inorganic fertilizers from the current levels of 5%. This will generate economic opportunities and improve the sustainability of the fertilizer industry.

In general, nutrient recycling of fish manure could be achieved via anaerobic digestion, composting or direct field application (Khiari et al., 2019). However, the key bottleneck in the recycling/valorisation of fish manure are the transportation to fields due to its high-water content and the of heavy metals concentration. In aquaculture, the traditional solid-liquid separation techniques consist of drum filters and posterior addition of coagulant and flocculants.

As mentioned before, there is a wide array of these chemicals, where the most common in aquaculture are aluminium sulphate, iron chloride (coagulants) and polyacrylamide type (flocculants). These metal-based chemicals and large chain polymers accumulate in sludge and eventually can have toxic effects to the biota, reducing their capacity to be used in further regeneration or reutilization processes. Iron chloride has been shown to have chronic effects on aquatic organisms, while aluminium has a direct effect on plant growth especially in acid soils. Furthermore, aluminium binds strongly with phosphorous reducing its bioavailability for reutilization as fertilizer.

3.1 Natural coagulant/flocculants

Natural coagulants or flocculants are derived from natural sources, such as plants, fungi and bacteria. These can be both ionic and non-ionic with various molecular weights. They are ecofriendly as they are biodegradable, normally produced from the residue of another process and generally nontoxic to aquatic biota and humans. Making them an alternative for substituting traditional coagulants/flocculants aiming at further recycling and reutilization of fish manure. Some of these examples are chitosan, tannins, cellulose, starch among others.

Cellulose, a natural flocculant, can effectively remove metal ions and organic debris from water. This is due to the abundance of free OH^+ groups on the linear polysaccharide chains, providing cellulose with a strong chelating impact (Fauzani et al., 2021). Traditionally, cellulose had limited use as a flocculant due to low solubility and chemical reactivity. However, studies using modified nano cellulose have shown to be effective in removing suspended matter (Fauzani et al., 2021). The addition of cellulose into the filter cake could then also assist in the flocculation of particles and improve overall filtration effects.

3.2 Lignocellulosic filtration

Lignocellulosic biomass is the most abundant biomass available on earth. It is produced from agricultural and forestry residues and can serve as a low-cost filtration material for the treatment of aquaculture wastewater. Lignocellulosic biomass included various agricultural residues (straws, hulls, stems, stalks) and deciduous and coniferous wood, have shown to be a good alternative as filtration material with a low cost and can be disposed in an environmentally friendly manner.

Lignocellulose is constituted by cellulose, hemicellulose, lignin, some proteins, oils and ash. It is used in the pulp and paper industry and to some extent in the production of bioenergy. Few studies have evaluated the capacity of lignocellulose as biosorbent to remove compounds from water as organic pollutants, heavy metals, inorganic compounds and microorganisms. Furthermore, lignocellulose base materials can go through thermal treatment to produce biochar.

3.3 Cake filtration and filter aids

Cake filtration is one of the different mechanical methods to separate particles from liquids and has the advantage that the resultant filter cake can be utilized for further posttreatments. Cake filtration uses porous a bed or porous media for treating high solid concentration suspensions, where smaller particles than the media (pore size) will be trapped, remaining in the filter surface forming bridges over individual opening of the filter medium. When these particles go through the separation process, they are captured both on the surface of the medium and the inner pore passages.

The system operation relies on the use of pressure on one side of the filter medium where the filtrate passes through the porous media/bed until the flow resistance is such that the filtration cycle can no longer continue at an economical rate, at which time the medium must be cleaned or replaced.

Filter aids increase the porosity of the cake which speeds up the filtration process through the reduction of specific cake resistance (Biller et al., 2018). This allows for a more extensive filtration of the suspension i.e. fish manure. There are two application methods, body feed filtration and precoating filtration. In body feed filtration, the filter aids can be added to the suspension directly prior to filtering. The particles in the suspension should adhere to the filter aid and form a lower specific cake resistance increasing filtration and quality of the filtrate (Heertjes and Zuideveld, 1978). In precoat filtration, a filter cake of precoat material is prepared on the filter mesh and particles are separated on and in the precoat cake. The precoat filter should be as open pored as possible to prevent clogging of the filter cake to utilize the depth effect of the filter cake (Bächle et al., 2021).

Lignocellulosic materials are suitable to be used ion cake filtration due to its fibrous structure which results in an open-pored cake and can be pumped. It was demonstrated that lignocellulosic biomass filter aids can increase the dry matter (DM) content to 25% in the filter cake compared to 5% in primary sludge (Biller et al., 2018). In addition, lignocellulosic materials have a high carbon/ organic matter content which can complement the N and P ratios of the fish manure for fertilizer or composting purposes. The relative proportion of carbon (C) to nitrogen (N) ratio for composting influences the process and quality of the compost. For general horticultural and agricultural use, the target C:N should be less than 20:1 and preferably below 18:1. If the C:N is higher than 20:1 it can lead to N immobilization (Rynk et al., 2021).

3.4 Research aims for cake filtration

The aim objectives of this part of the study were to identify the optimal amount and composition of lignocellulose and cellulose needed for a precoat filter cake to:

- Effectively and efficiently filter a high volume of aquaculture sludge with a high dry matter content of fish sludge beyond 25%
- Determine the C:N:P ratio are sufficient for the filter cake to be used as fertilizers and soil amendments.

3.5 Materials and methods fish manure cake

3.5.1 Feedstock for Cake Filtration Precoat

The lignocellulose used for the precoat feedstock was LIGNOCEL® developed by J. Rettenmaier & Söhne GMBH + Co KG, Germany. The cellulose product with a particle size of 180µm, made from perennial plants, is also developed by the same company. The proximate composition for lignocellulose and cellulose is shown in Table 16.

Table 16. Proximate composition analysis of feedstock for precoat.

	Lignocellulose	**Cellulose**
Dry Matter (%)	95.3	99.4
Ash (%)	0.5	98.2
Organic Matter (%)	94.8	1.2
Total Nitrogen (%)	0.036	0
Total Phosphorus (%)	0.005	0.014

3.5.2 Cake filter composition combinations

Cake combinations of 20g, 25g, 30g and 35g of lignocellulose with 2g of cellulose were tested to determine the filtration capacity in relation to the amount of lignocellulose used. The amount of lignocellulose with the best filtration capacity was then selected for further optimization by changing the amount of cellulose used. Thus, cake combination of 35g of lignocellulose with 0.5g, 0.75g and 1g of cellulose were tested to determine the optimal lignocellulose and cellulose combination for 1 L of sludge at a TCOD of 4.85g /L. All combinations were conducted in triplicates.

A control was also conducted with a cake combination of 35g of lignocellulose with 2g of cellulose where only 1 L of milli-Q water was filtered through.

3.5.3 Lab scale experiment design and set-up

A Büchner funnel was placed on a 2.5L Büchner flask that is connected to a rubber tubing with a vacuum pump set at 2 psi. Filter paper of 12-15 µm is placed in the Büchner funnel. The dry materials for each cake filter combination were weighed out and thoroughly mixed in a beaker. 40ml of milli-Q water was added to the dry mix to increase the cohesiveness of the precoat. Once evenly wet, the precoat was poured out on the filter paper in the ceramic funnel and lightly pressed down with a spoon to create an even layer.

The diluted sludge was thoroughly mixed and segregated into 1L batches. Prior to the filtration, the diluted sludge was gently shaken to homogenize the suspension without breaking the particles. The diluted sludge is then slowly poured over the precoat in a circular manner to evenly utilize the precoat. The diluted sludge was poured gradually, and stopping periodically if filtration was slow, for a maximum of 10 minutes. After the set time, the pump was stopped, and the rubber tubing was disconnected. The filter cake was then homogenized with a spoon before transferring it into a container.

All filter cakes were stored at -20 °C prior to analysis for dry matter (ISO 6496, 1983), ash (ISO 5984, 1978), total Kjeldahl Nitrogen (ISO 5983-2,2005), and total phosphorus (ISO 6491,1998). Three 15 ml samples of the filtrate were collected and filtered and/or preserved for TCOD, SCOD and Anions as mentioned in the above section. TCOD and SCOD were determined using digestion vials (LCK 314, 514, and 1414, Hach Lange, Germany). Anions were analyzed with an ion chromatography (930 Compact IC Flex 1 with a Metrosep A Supp 7- 250/4.0 column combined with a 887 Professional UV/VIS detector Metrohm, Sweden) with 0.1M H_2SO_4 as suppressor and 3.6mM Na_2CO_3 as eluent.

3.5.4 Pilot scale fish manure cake

Once the technology has been evaluated and optimized in laboratory conditions, the optimal ratio between cellulose and lignocellulose was used to further evaluate a pilot scale fish manure treatment.

The system consists of a 50L mixer where the lignocellulose+cellulose and fish sludge are added and mixed at 250 rpm. The mixture ratio is 35 g of lignocellulose + 6 g of cellulose per liter of fish sludge treated. Once the mixture is homogenized (approx. 5 min) a double diaphragm displacement air driven pump (Wilden 11/2” P400) moves the mixture into a pressurized filtration chamber (21L). The pressurized chamber has 5 filtration tubes with a

60 µm mesh. Water passes through the filtration tubes while the mix of fish manure, lignocellulose and cellulose are retained in the filtration tubes. Once the inner pressure reaches 3 bars and water comes out from the vent valve, the recirculation loop is activated. Water is then recirculated between the 50L mixer and the filtration system. After 15 min of operation, water coming out of the filter is clean and water is discharged from the system.

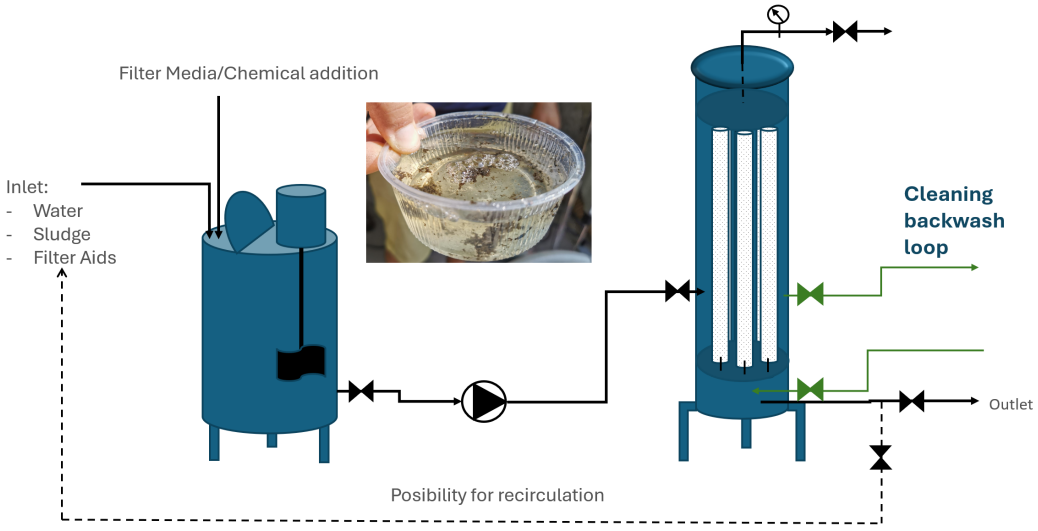

Figure 39. Scheme of pilot scale plant for production of fish manure cake.

The system was evaluated for the treatment of freshwater RAS from Christiansminde and saltwater sludge at DTU Aqua.

3.5.5 Data analysis

$$\boldsymbol{Weight\ of\ cake\ without\ lignocellulose\ and\ cellulose} = \boldsymbol{Weight\ of\ cake} - \boldsymbol{Weight\ of\ lignocellulose\ and\ cellulose}$$ (Equation 14)

$$* \boldsymbol{TCOD\ filtered}\ (\boldsymbol{mg}) = \boldsymbol{TCOD\ of\ diluted\ sludge}\ (\boldsymbol{mg/L}) \times \boldsymbol{amount\ of\ sludge\ filtered}\ (\boldsymbol{L})$$ (Equation 15)

$$* \boldsymbol{TCOD\ in\ filtrate}\ (\boldsymbol{mg}) = \boldsymbol{TCOD\ of\ filtrate}\ (\boldsymbol{mg/L}) \times \boldsymbol{amount\ of\ filtrate}\ (\boldsymbol{L})$$ (Equation 16)

$$* \boldsymbol{TCOD\ removed\ by\ cake\ filter} = \boldsymbol{TCOD\ filtered}\ (\boldsymbol{mg}) - \boldsymbol{TCOD\ in\ filtrate}(\boldsymbol{mg})$$ (Equation 17)

*Same equations for SCOD and xCOD.

3.5.6 Mass loading equations

$$\boldsymbol{TP\ in\ Sludge}\ (\boldsymbol{mg}) = \boldsymbol{organic\ P\ in\ fish\ cake}\ (\boldsymbol{mg}) + \boldsymbol{PO4-P\ in\ filtrate}\ (\boldsymbol{mg})$$ (Equation 18)

$$\boldsymbol{TN\ in\ Sludge}\ (\boldsymbol{mg}) = \boldsymbol{organic\ N\ in\ fish\ cake}\ (\boldsymbol{mg}) + \boldsymbol{NO2-N\ in\ filtrate}\ (\boldsymbol{mg}) + \boldsymbol{NO3-N\ in\ filtrate}\ (\boldsymbol{mg})$$ (Equation 19)

$$\boldsymbol{OM\ in\ Sludge}\ (\boldsymbol{g}) = \boldsymbol{organic\ matter\ in\ fish\ cake}\ (\boldsymbol{g}) + \boldsymbol{TCOD\ in\ filtrate}\ (\boldsymbol{g})$$ (Equation 20)

3.6 Results fish manure cake, lab. scale

More sludge could be filtered out with precoats with at least 30g of lignocellulose (Fig. 40). For the same amount of sludge filtered, the precoat with 35g of lignocellulose had a lower filter cake weight as compared with a precoat of 30g of lignocellulose (Fig. 41). This suggests that 35g of lignocellulose was the most optimal to filter out 1L of sludge at a TCOD of 4.85g/ L.

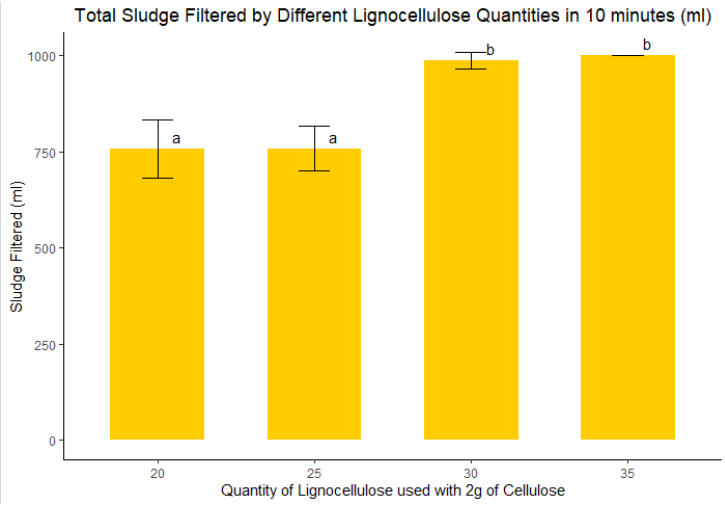

Figure 40. Amount of sludge filtered (max 1 litre) in 10 minutes for different lignocellulose quantities with 2g of cellulose (mean ± standard deviation, n=3).

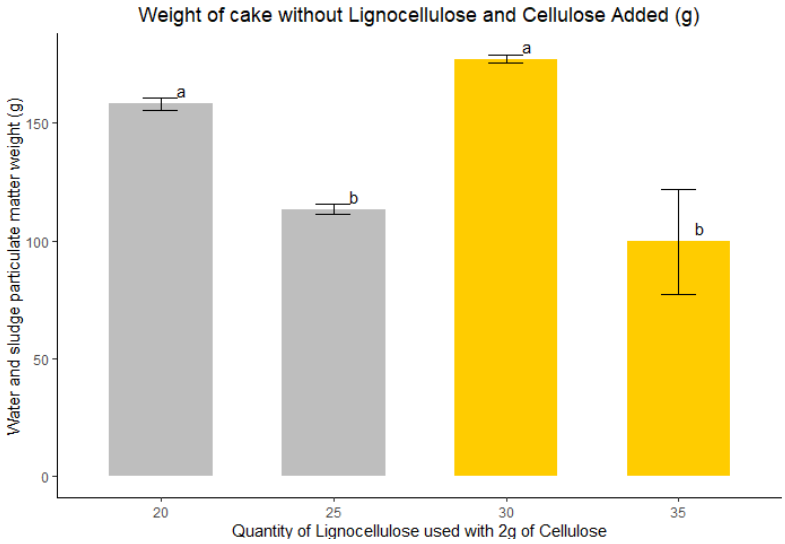

Figure 41. Weight of the water and sludge particulate matter for different lignocellulose quantities with 2g of cellulose (mean ± standard deviation, n=3), bars in grey to signify that these combinations filtered out less than 1L of sludge and thus not comparable to those that did.

All cake combinations using 35g of lignocellulose with 0.5g to 2g of cellulose could filter out 1L of sludge within 10 minutes. However, filter cakes with 1g to 2g of cellulose had a lower water and sludge particulate matter weight as compared to filter cakes with only 0.5g of cellulose (Figure 42). While the filter cake with 0.75g of cellulose was not different to all the

other combinations. The average weight of the filter cake with 35g of lignocellulose and 0.75g of cellulose is 192.7 ± 53 g.

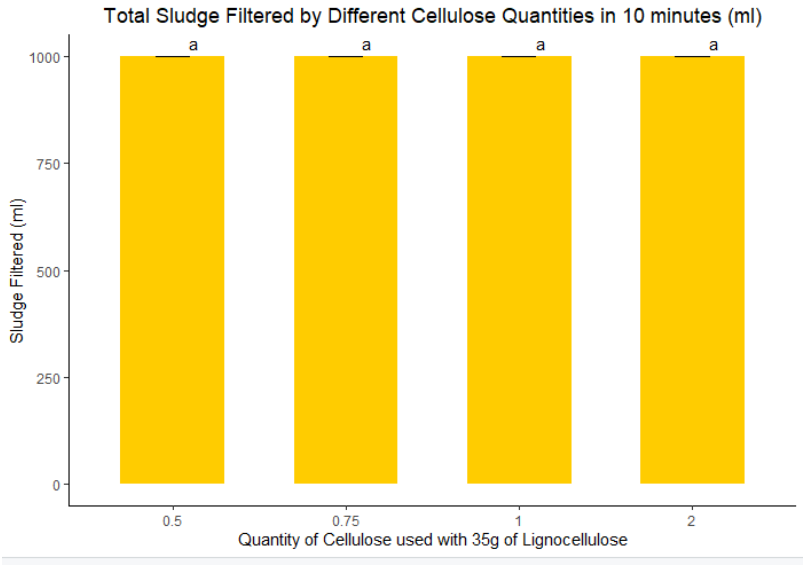

Figure 42. Amount of sludge filtered (max 1 litre) in 10 minutes for different cellulose quantities with 35g of lignocellulose (mean ± standard deviation, n=3).

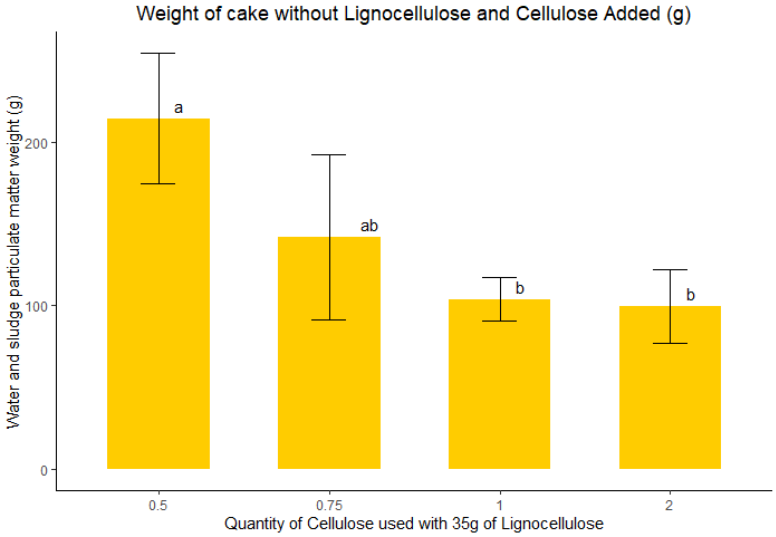

Figure 43. Weight of the water and sludge particulate matter for different cellulose quantities with 35g of lignocellulose (mean ± standard deviation, n=3). Significance was set at P <0.05 and letters denote significant differences between treatments.

Cakes containing lower than 35g of lignocellulose and lower than 0.75g of cellulose were too moist and were not analyzed. As such, all results reported here forth refers only to cake filters containing 35g of lignocellulose combined with 0.75, 1 and 2g of cellulose. They will be referred to as C0.75, C1 and C2 respectively. All cake filters minimally had 17% dry

matter (DM) and there was no difference in DM, ash, organic matter, and TN content between the different cellulose treatments (Table 17). Only TP content was lower in the combination of 35g lignocellulose and 2g cellulose.

Table 17. Proximate composition of filter cakes using 35g of lignocellulose and varying cellulose weights from 0.75g to 2 g.

Parameters	C0.75 (0.75gCellulose)	C1 (1gCellulose)	C2 (2gCellulose)	One- way ANOVA p-value
DM (%)	24.1 ± 5.88	29.0 ± 2.02	29.0 ± 3.48	0.307
Ash (%)	1.66 ± 0.42	2.19 ± 0.12	2.14 ± 0.31	0.149
Organic Matter (%)	22.4 ± 5.46	26.8 ± 1.93	26.9 ± 3.22	0.328
TN (%)	0.106 ± 0.02	0.120 ± 0.01	0.144 ± 0.02	0.071
TP (%)	0.210 ± 0.06	0.256 ± 0.02	0.114 ± 0.01	**0.007**

As reflected in Fig. 44, C2 cake filter filtered out a higher amount of TCOD compared to the other treatments (P = 0.0017). The filtrate of C2 had lower amounts of SCOD (P = 1.6e-3), which meant it had a higher amount of xCOD (P = 2.3e-5).

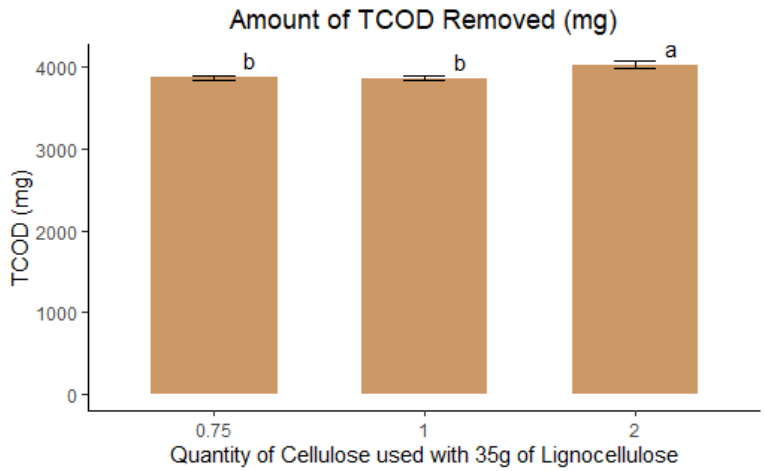

Figure 44. Mass of TCOD removed under different cellulose to lignocellulose combinations.

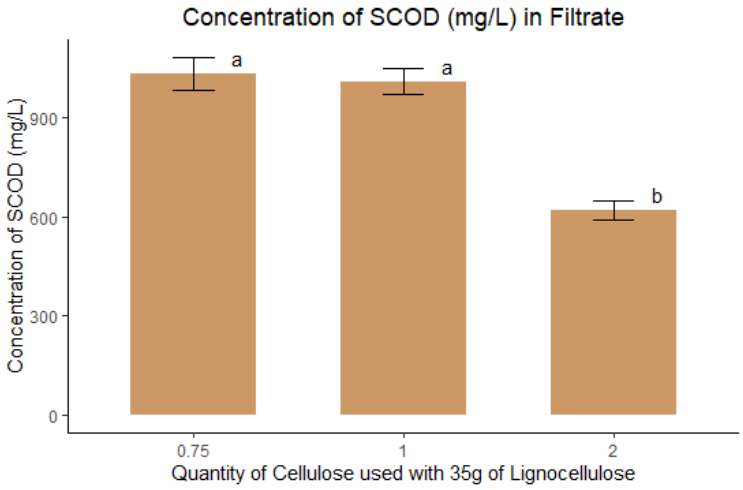

Figure 45. Concentration of sCOD removed under different cellulose to lignocellulose combinations.

The C0.75 and C2 cake filter resulted in the highest reduction of NOx-N concentration in the filtrate (P = 0.0145) (Fig. 46).

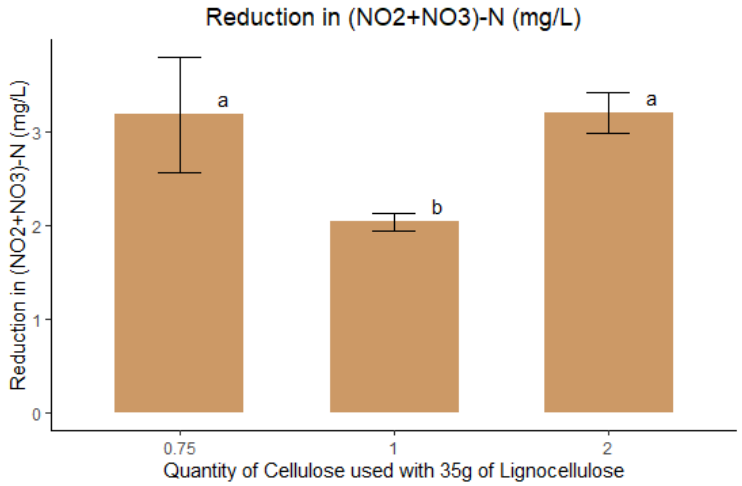

Figure 46. Nitrate and nitrite removed under different cellulose to lignocellulose combinations.

While all filter cakes resulted in a higher PO_4-P concentration in the filtrate compared to the diluted sludge, C2 treatment had the lowest increase across the treatments (P = 5.1e-5) (Fig. 47).

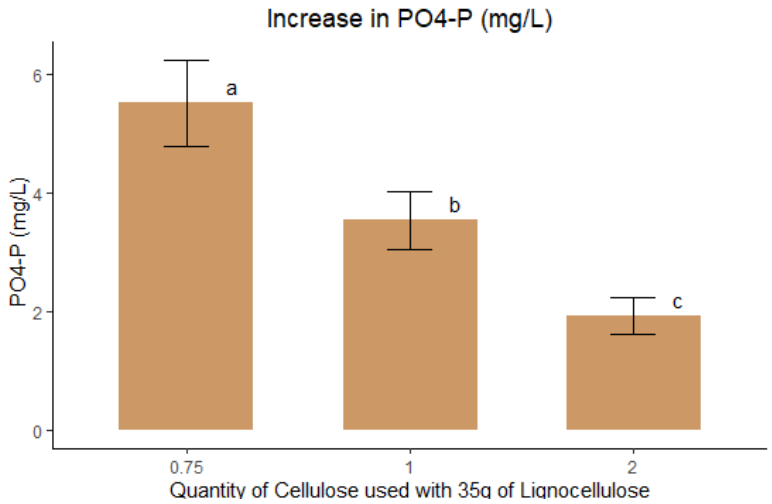

Figure 47. Phosphorous removed under different cellulose to lignocellulose combinations.

3.6.1 Mass nutrient retention on fish manure cake

When comparing the filtrate from the control filter cakes[1] with the filtrate of the C2 filter cake, it showed that 9%, 0.5% and 7.5% of NO_3-N, NO_2-N and PO_4-P came from the pre-coat.

Most of the organic matter in the C2 cake filter was from the precoat (i.e. lignocellulose and cellulose) with only 8% from the sludge. The mass balance for C2 filter cake shows that more than 75% of the nitrogen, phosphorus and organic matter were captured in the fish cake (Fig. 48, 49, 50 and 51).

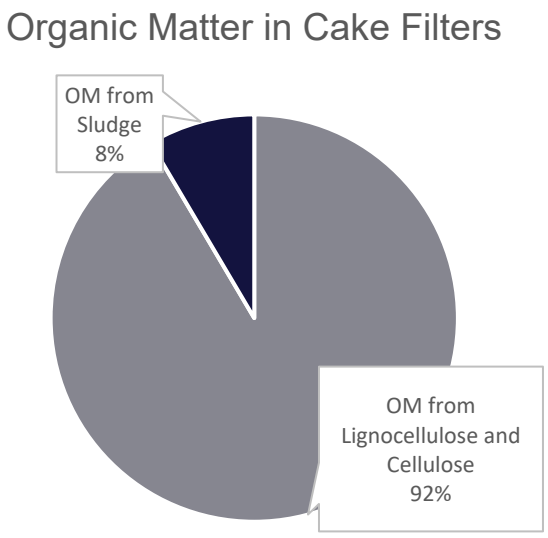

Figure 48. Organic matter composition in the fish manure cake.

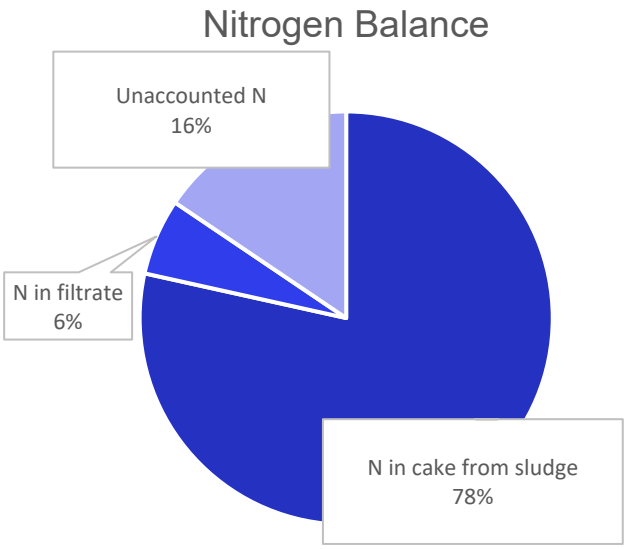

Figure 49. Distribution of the nitrogen mass retained in the fish manure cake.

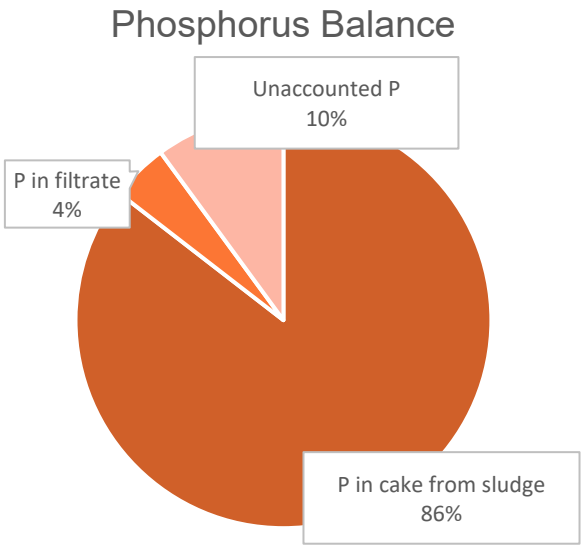

Figure 50. Distribution of the phosphorous mass retained in the fish manure cake.

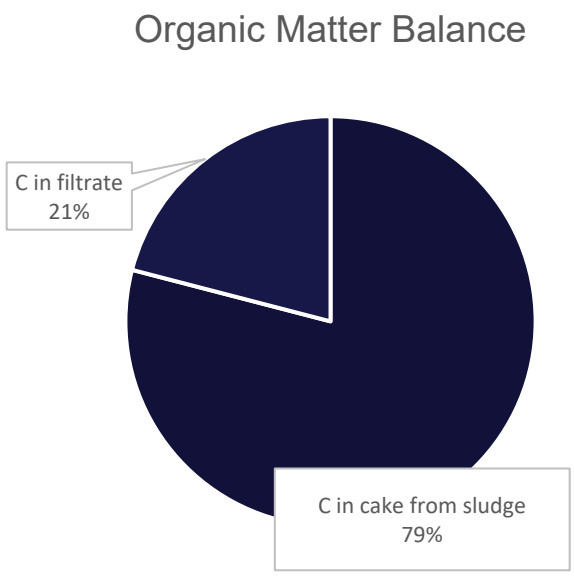

Figure 51. Distribution of the organic matter mass retained in the fish manure cake.

The higher the amount of lignocellulose and cellulose provided in the cake, the greater the volume of manure filtered and the higher the dry matter content of the filter cake. The higher amount of lignocellulose in the precoat provided more filtering capacity before clogging. This is likely due to the increased void volume of the cake with more lignocellulose material i.e. more volume.

While the amount of cellulose did not affect the amount of manure that could be filtered, it affected the weight of the liquid retained in the cake. This means the quantity of lignocellulose used determined the filtering capacity of the cake instead of cellulose. As a natural flocculant, a higher cellulose content may have flocculated the sludge particles, narrowing the particle size distribution in the manure, and increasing the particle size. It is known that lower filter cake resistance is related to a large particle size as well as the porosity (Hennemann et al., 2021). The narrower the size distribution of the particles, the higher the porosity and thus lower filter cake resistance (Hennemann et al., 2021). As such, a higher amount of cellulose would lower filter cake resistance and increase the flow rate of the filtrate through the cake, making filter cakes with more cellulose lighter. In addition, it was observed that filter cakes with more cellulose had a more stable filter cake structure which could have made the filter cake more porous and open during filtration.

The results indicate that a minimum ratio of 0.75g to 35g of lignocellulose is required to retain sufficient cake structure during filtration and it can be concluded that this is the optimal filter cake composition to filter out 1L of sludge at a TCOD of 4.85g/L. This combination can effectively reduce 1L of sludge to an average weight of 177g. This equates to >80% reduction in weight and likely significant savings in operational cost for the farms. The dry matter results (for filter cakes with 35g of lignocellulose) averaged at 24 to 29%. This was similar to previous studies reporting 25% dry matter (Biller et al., 2018).

3.6.2 Results Pilot scale Fish manure cake

The pilot scale fish manure technology was tested at Christiansminde for the freshwater trials and at DTU aqua Campus Hirtshals for the saltwater trials.

Figure 52: Pilot scale fish manure cake system.

Results from the pilot scale fish manure technology show that the technology can be upscaled with positive results, reaching a high dry matter content and accumulation of N and P.

Table 18. Proximate composition of fish cake manure n=8.

	Fish Manure Cake
Dry matter %	20.2±3.1
P (g/kg)	0.8±0.5
N (g/kg)	1.2±0.7

Furthermore, and analysis of heavy metals contained in the fish sludge showed that by utilizing the fish manure cake technology the concentrations of this heavy metals is significantly reduced as compared to the limits stablished by Danish legislation (max. levels in brackets). In this case we can see that Cadmium and Nikel levels found in fish sludge can be significantly reduced utilizing the proposed technology.

Table 19. Comparison on heavy metal composition between fish sludge and fish manure cake.

Metals	
Farm	**Fish manure cake**
Cadmium 1.1 mg/kg (**0.8**)	0.047 mg/kg
Nikkel 29 mg/kg (**30**)	0.22 mg/kg

Because the C:N:P ratio was shown to be too low for considering the product as a fertilizer, the fish cake was tailored by adding struvite, a solid residue from wastewater treatment plants which composition is ammonium magnesium and phosphorous. In table 20 we can see the different composition of the ingredients used for the fish manure cake and the final composition of the fish manure cake.

Figure 53. Struvite material used for enhancing P and N in fish manure cake.

Table 20. Proximal composition of materials used to tailor the fish manure cake.

Tailoring the composition					
	Sludge	**Struvite**	**Lignocellulose**	**Cellulose**	**Cake**
Dry matter %	0.5	57	99	95	32
P (g/kg)	0.3	125	0	0	20
N (g/kg)	0.45	56	0.36	0	9.55

Effect of different lignocellulosic inclusions on the fish manure cake

To evaluate the effect of different levels of lignocellulose inclusions (0.5, 1.0 and 1.5 kg) on the production and composition of fish cake, different trials were performed.

Saltwater trials with and without coagulant

The trial consisted of treating saltwater sludge from RAS with and without use of coagulant. The results show that for the three levels of inclusion without the use of coagulant the removal efficiency ranged between 78-84% for N and between 79-83% for P, while the dry matter ranged between 234-275 g/kg. There is slight a correlation between P removal and increased lignocellulosic material, most probably related to the fact most of the P fraction is contained in the sludge, while N species will mostly be present in a dissolved fraction (liquid).

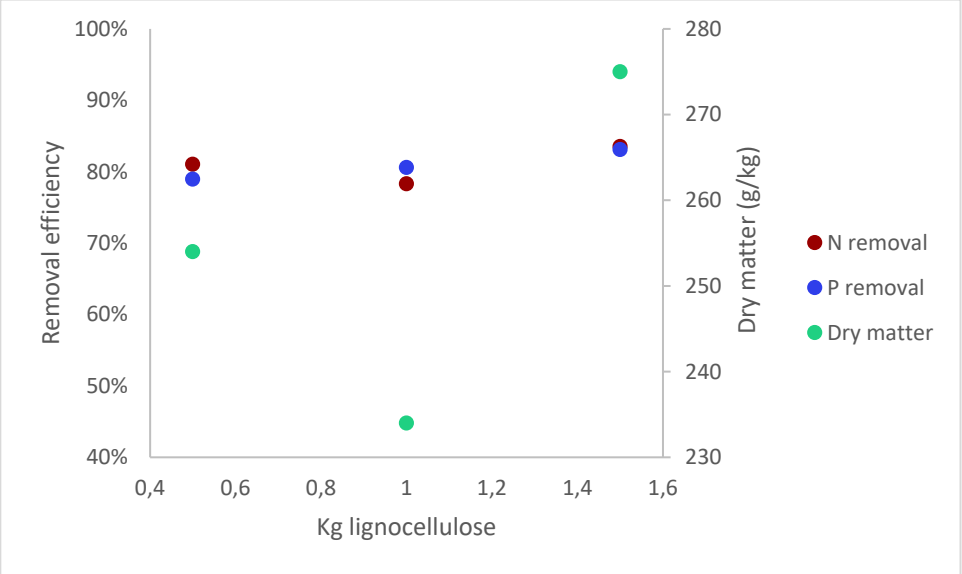

Figure 54. Nitrogen and phosphorous removal efficiency at different levels of lignocellulose obtained from a saltwater RAS.

Regarding the N and P content of the fish manure cake, as the same amount and type of sludge was used in the trials, we can see that as lignocellulose applied is reduced (0.5 kg) the content of N and P in the final cake is increased as compared to higher lignocellulose application, while obtained dry matter does not change significantly. This will allow us to tailor the C:N:P ratio and reduce the C content for improving the composition for composting purposes.

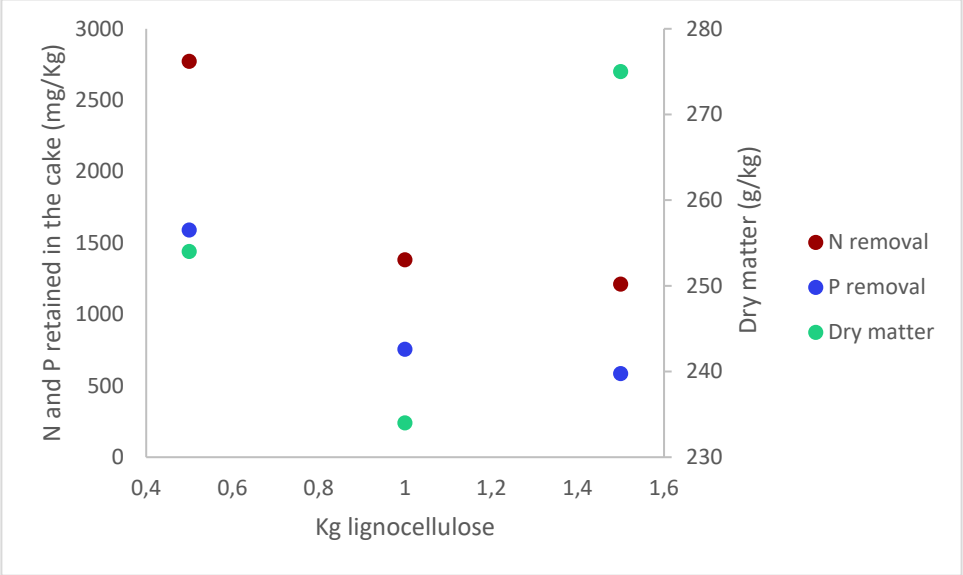

Figure 55. Nitrogen and phosphorous retained in cake at different levels of lignocellulose obtained from a saltwater RAS.

The addition of coagulant has a direct effect on the P and N removal efficiency. Without coagulant P removal reached a maximum of 83% while with the use of coagulant the P removal capacity was between 91-95%. In the case of N the removal capacity was also enhanced with the use of coagulant. Without coagulant the max removal capacity was 84% while with the use of coagulant the removal capacity ranged between 84-88%.

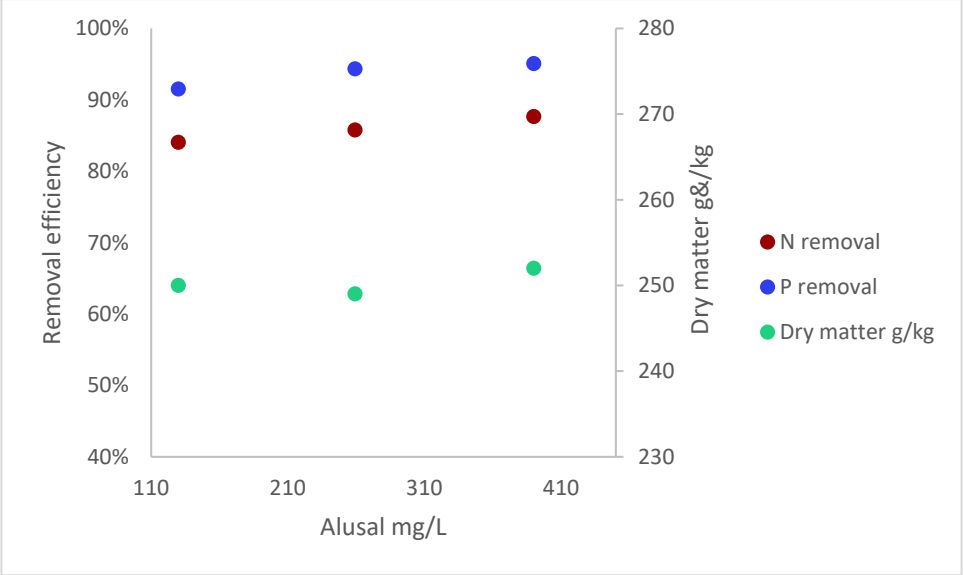

Figure 56. Nitrogen and phosphorous removal efficiency at different levels of Alusal (coagulant) obtained from a saltwater RAS.

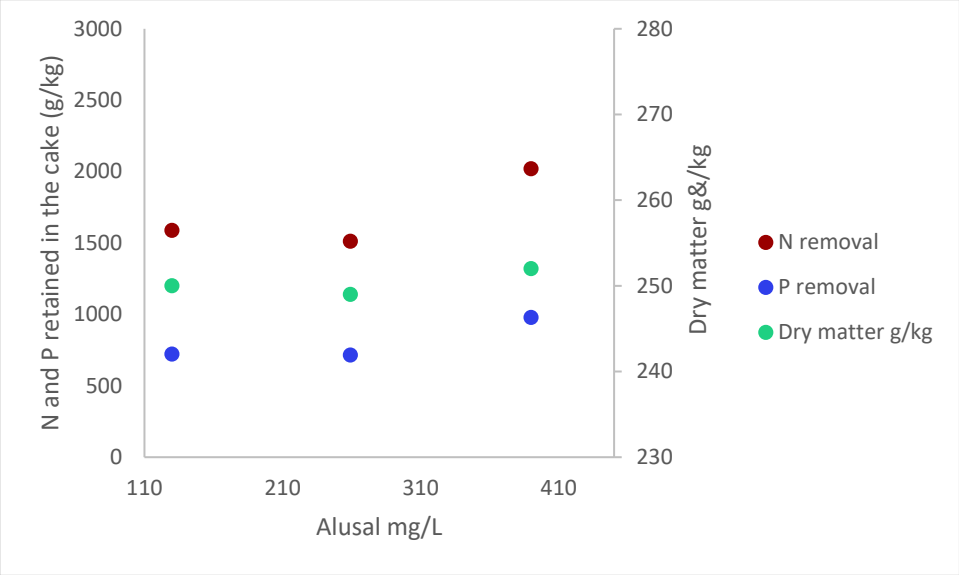

Figure 57. Nitrogen and phosphorous retained in cake at different levels of Alusal (coagulant) obtained from a saltwater RAS.

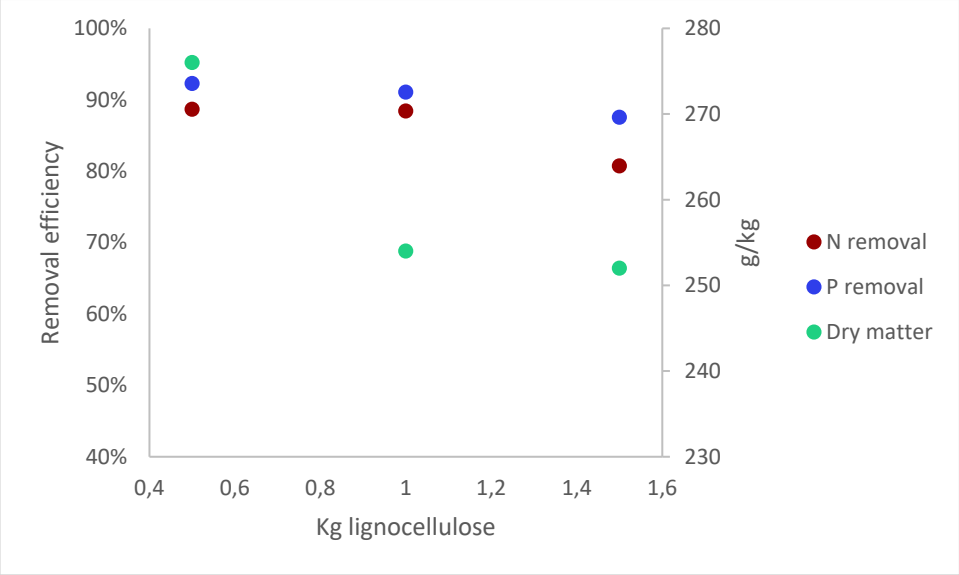

Figure 58. Nitrogen and phosphorous removal efficiency at different levels of lignocellulose obtained from a freshwater RAS.

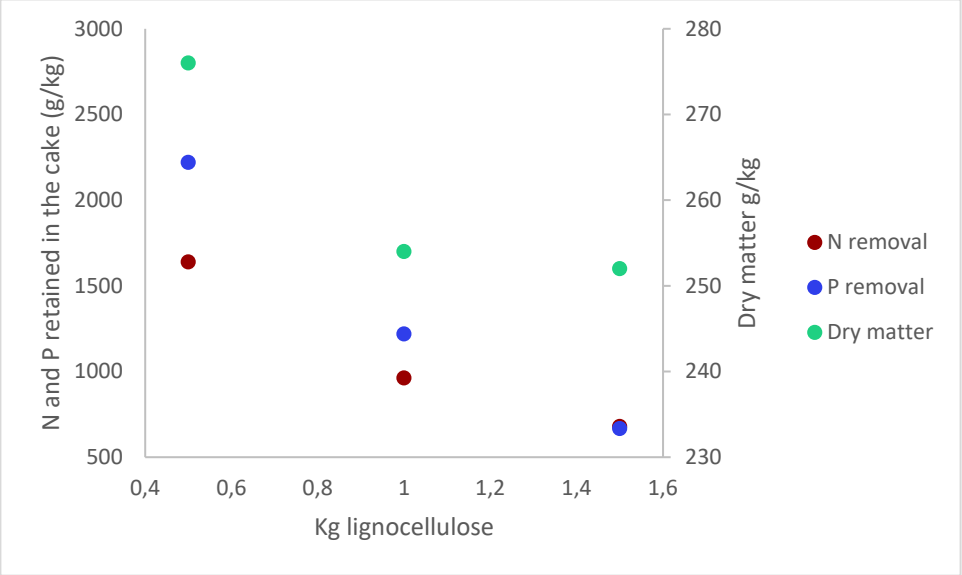

Figure 59. Nitrogen and phosphorous retained at different levels of lignocellulose obtained from a freshwater RAS sludge.

3.6.3 Valorization of fish manure cake

To validate the capacity of the fish manure cake for being a growing substrate, different levels of inclusion were used for growing mushrooms. Two levels of inclusion (15 and 30%) of fish manure cake plus a control (flour) in fresh and saltwater conditions were seeded with mushroom mycelium.

The results showed that in all freshwater treatments it was possible to grow mushrooms using the fish cake manure. In the case of saltwater fish manure treatments there was a reduced growth as inclusion of saline material increased.

Demonstrating that the fish manure cake is a viable substrate for growing mushrooms from freshwater material. In the case of saltwater material even though there were growth further experiments are required to elucidate which is the best inclusion level to apply.

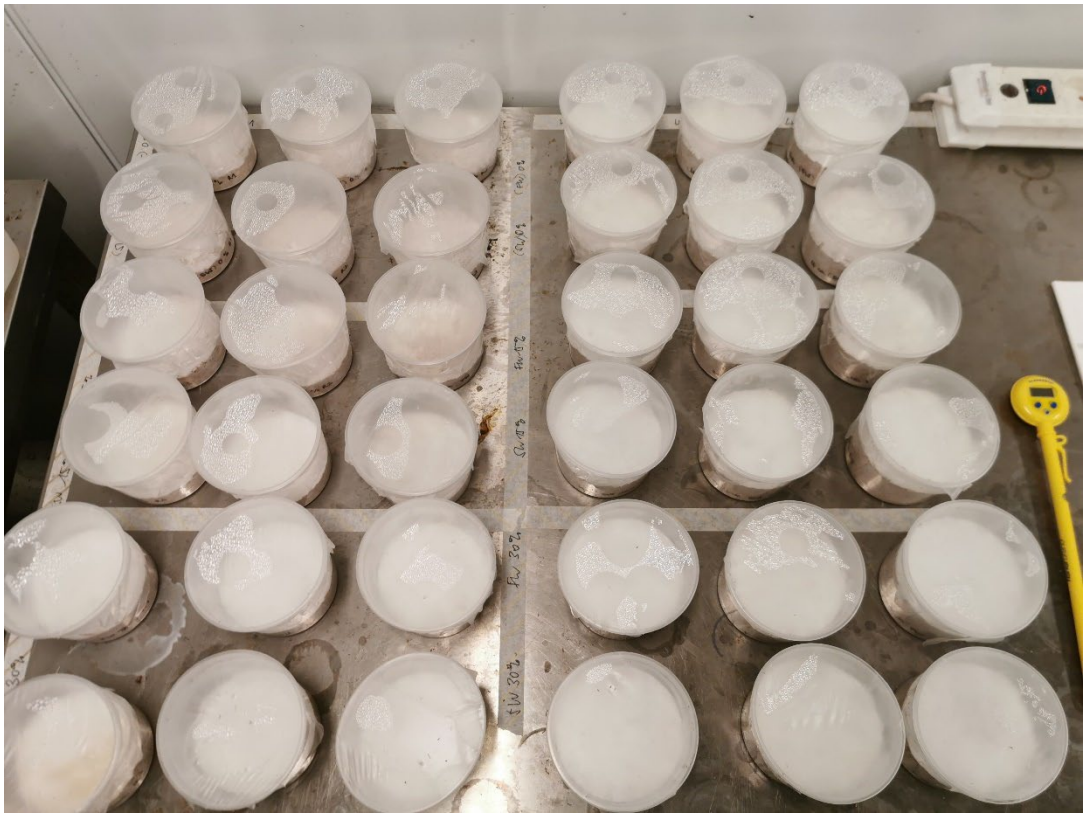

Figure 60. Valorization trials on growing mushrooms at different salinities and inclusion levels using fish manure cake.

Figure 61. Valorization trials on growing mushrooms at different salinities and inclusion levels using fish manure cake.

Figure 62. Valorization trials on growing mushrooms at different salinities and inclusion levels using fish manure cake.

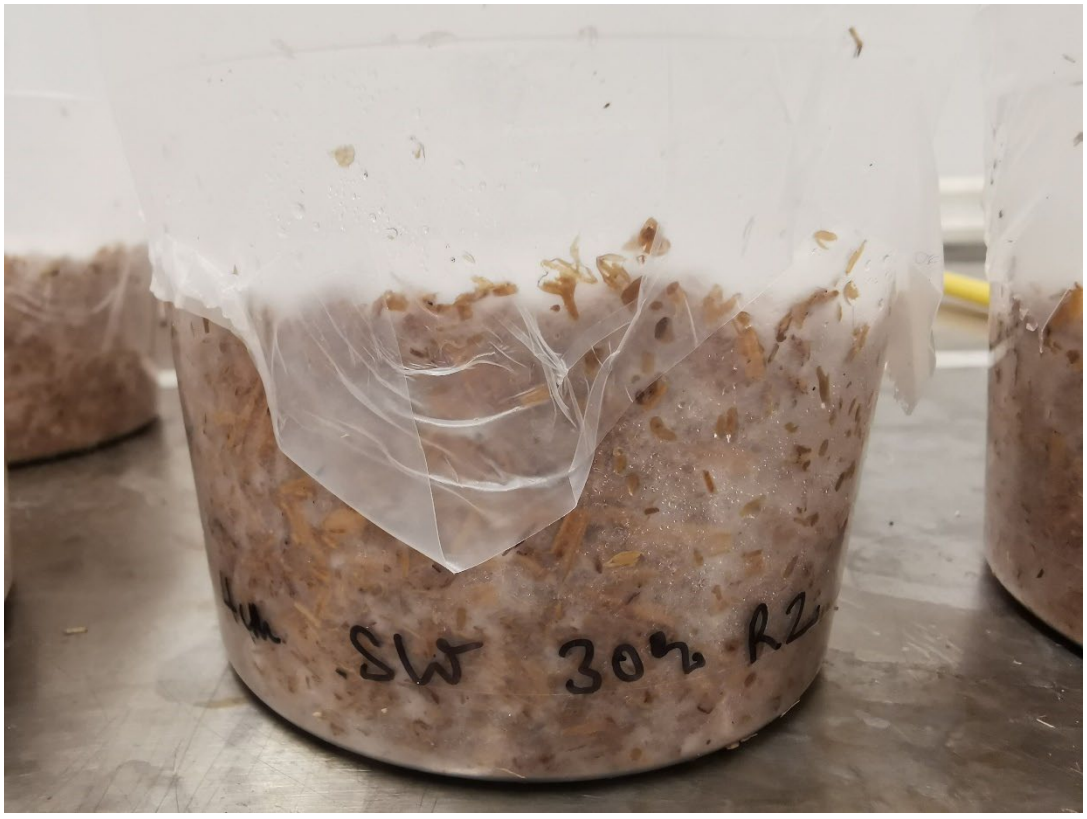

Figure 63. Valorization trials on growing mushrooms at different salinities and inclusion levels using fish manure cake.

3.7 Summary and future development on fish manure cake

The proposed technology for valorizing fish manure proved to have several positive aspects such as water reduction, increased dry matter, significant reduction of heavy metals and the possibility to increase value for saline sludge. Because of the high dry matter achieved the material can be utilized for biochar production or eventually in biogas plants.

The technology has the potential to tailor the fish manure cake composition by adding, for example struvite which increased the N and P content of the material, reaching levels similar to fertilizers giving fish manure an additional value

Further studies are required to scale up the technology, improve filtration capacity and automatize the collection for the fish manure cake. Additionally it will be interesting to evaluate alternative materials for creating the filtration bed that have lower or close to cero commercial value as the lignocellulose applied in this study.

All in all, the technology shows a huge potential for valorizing fish manure while reducing the collection and treatment of this residual resource.

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