Transforming waste into new resources: optimizing sludge hydrolysis to improve nitrogen removal in aquaculture through denitrification

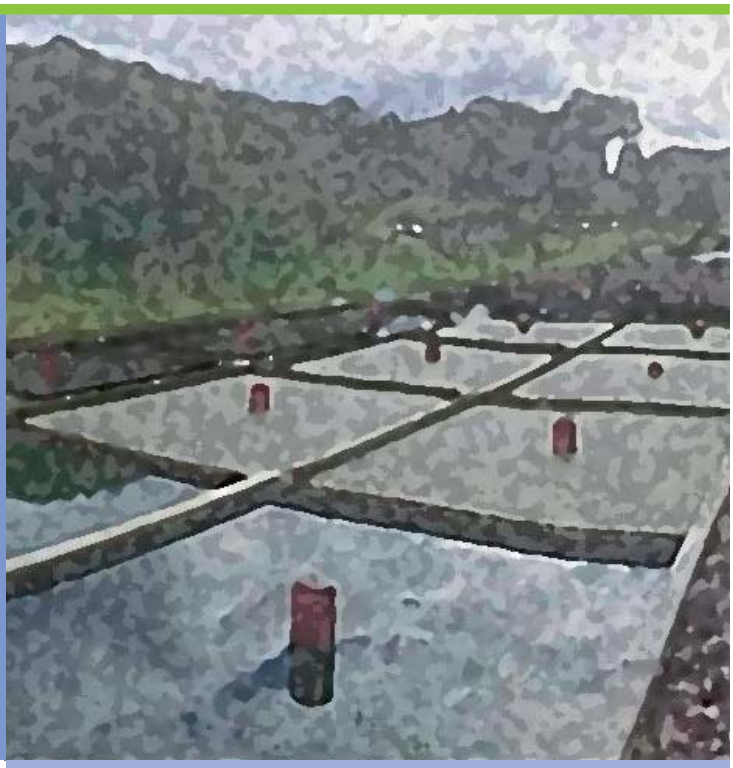
Letelier-Gordo, Carlos Octavio; Pedersen, Per Bovbjerg; Dalsgaard, Anne Johanne Tang

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

Transforming waste into new resources: optimizing sludge hydrolysis to improve nitrogen removal in aquaculture through denitrification

PhD Thesis



Written by Carlos O. Letelier Gordo
Defended 10 February 2017

DTU Aqua
National Institute of Aquatic Resources

Transforming waste into new resources: optimizing sludge hydrolysis to improve nitrogen removal in aquaculture through denitrification

PhD thesis by
Carlos O. Letelier Gordo
November, 2016
Hirtshals, Denmark

Technical University of Denmark
National Institute for Aquatic Resources
Section of Aquaculture

Preface

This *Ph.D.* dissertation is submitted in partial fulfillment to attain the Doctor of Philosophy degree (*Ph.D.*). The work shown herein was undertaken during my enrollment as *Ph.D*. student at the Section for Aquaculture, National Institute of Aquatic Resources (DTU Aqua), Technical University of Denmark, in Hirtshals, Denmark.

I would like to thank my supervisor Per Bovbjerg Pedersen for his total trust and confidence on my work, being always available to clear my existential scientific doubts.

My co-supervisor Dr. Anne Johanne Tang Dalsgaard for her critical review of my work, helping me to improve considerably my way of presenting the data and bringing me back to earth from my esoteric discussions.

Thank you Ulla Sproegel, Brian Møller, Sara M. Nielsen and Melissa Lyng for the nourishing lab method discussions, the interest on the trial results and interpretations, but most of all, thanks for always receiving me with a smile.

Thanks to all the people in the Institute that made my stay worthy and unforgettable.

My family, my mother and grandfather, this is for you. Thank you.

Hirtshals, 15 November, 2016.

"Si no creyera en lo que agencio
si no creyera en mi camino
si no creyera en mi sonido
si no creyera en mi silencio.
que cosa fuera la maza sin cantera
un amasijo hecho de cuerdas y tendones
un revoltijo de carne con madera
un instrumento sin mejores resplandores
que lucecitas montadas para escena"

Silvio Rodriguez, La Maza.
(Silvio, 1982)

Contents

List of abbreviations

Abbreviation	Description	Unit
ΔG^0	Charge in Gibbs free energy	kJ/mol
ADM1	Anaerobic digestion model 1	
ASBR	Anaerobic sequence batch reactor	
BOD	Biological oxygen demand	mg O_2/L
C:N	Carbon to nitrogen ratio	g/g
COD	Chemical oxygen demand	mg O_2/L
d	Day	day
EOP	End-of-pipe treatment	
Eth	Ethanol	gCOD
h	Hour	hour
HAc	Acetic acid	gCOD
HBu	Butyric acid	gCOD
HFo	Formic acid	gCOD
HPr	Propionic acid	gCOD
HRT	Hydraulic retention time	d^{-1}
HVa	Valeric acid	gCOD
MTF	Model trout farm	
NFE	Nitrogen free extracts	mg/L
NH_4^+-N	Ammonium nitrogen based	mg/L
NO_2^--N	Nitrite nitrogen based	mg/L
NO_3^--N	Nitrate nitrogen based	mg/L
P:E	Protein to energy ratio	g/MJ
PO_4^{3-}-P	Orthophosphate P based	mg/L
PO_4^{3-}-P/TP	phosphorous solubilization	g/g
Q	Flow	m^3/d
RAC	Readily available carbon source	gCOD
RAS	Recirculating aquaculture system	
SBM	Soybean meal	
sCOD	soluble chemical oxygen demand	mgO_2/L
sCOD/TCOD	Degree of solubilization	g/g
SFS	Settable faecal solids	
SS	suspended solids	
SSF	Side stream fermenter	
TAN	Total ammonia nitrogen	mg/L
TAN/TKN	Nitrogen solubilization	g/g
TCOD	Total chemical oxygen demand	mgO_2/L
TOC	Total organic carbon	mg/L
TKN	Total Kjeldahl nitrogen	g/L
TN	Total nitrogen	g/L
TS	Total solids	g/L
TVS	Total volatile solids	g/L
v/v	volume to volume	
VFA	Volatile fatty acid	g/L
VFA_COD/sCOD	Degree of fermentation COD based	g/g

Dansk populært resume

Miljømæssige begrænsninger har I Danmark tvunget akvakultursektoren til at forbedre såvel drift som vandrensningsteknologier således at sektoren er blevet stadig mere miljømæssig bæredygtig. Selv om erhvervet har reduceret sin specifikke udledning (kg/t produceret fisk) af alle næringsstoffer (N, P og O) er kvælstoffjernelse stadig den største hindring for en egentlig udvidelse af produktionen. 15 – 50 % af kvælstoffet kan typisk fjernes ved anvendelse af bedst tilgængelige teknologi, herunder plantelaguner i Modeldambrug type III.

Udledningen af kvælstof fra recirkulerede anlæg (RAS) består langt overvejende af nitrat-kvælstof (NO_3^--N), idet nitrat er resultat af omdannelsen af ammonium (NH_4^+) i de biologiske filtre. Fiskenes udskillelse af kvælstof sker langt overvejende som ammonium, som via mikrobiologisk oxidation (nitrifikation) omdannes til nitrat. Nitratfjernelse fra afløbsvandet er derfor et vigtigt skrift at tage såfremt udledningen af kvælstof skal reduceres.

Heterotrof denitrifikation – en biologisk proces hvorunder bakterier reducerer nitrat (NO_3^-) til frit kvælstof (N_2) under forbrug af organisk kulstof som elektron donor (energikilde) – er central for en effektiv kvælstoffjernelse. Slammet produceret af fiskene i anlægget vil kunne bruges som intern kulstofkilde til denne proces, hvorved to typer "affald" – slam og kvælstof – kan behandles samtidigt og udledningen af begge reduceres. Samtidigt kan eventuelle udgifter til indkøb af ekstern kulstofkilde (f.eks. metanol el. ethanol) og slamtransport reduceres.

Foder er (indirekte) kilden til al affaldsproduktion på recirkulerede anlæg, idet mængde, sammensætning og fordøjelighed af foderet afgør mængder og sammensætning af det producerede affald kaldet produktionsbidraget. En kobling af foderet med produktionsbidraget og slammets muligheder som intern kulstofkilde er derfor såvel oplagt som nødvendig.

Denne PhD afhandling fokuserer på mulighederne for anvendelse af slam produceret af fiskene som intern kulstofkilde til denitrifikation, hvorved organisk affald så at sige transformeres til en ny ressource i linie med det såkaldte "Residual Resource" princip. Omdannelsen fra partikulær slam til anvendelig, opløst kulstofkilde foregår via to koblede, mikrobiologiske processer: hydrolyse og fermentering. Under hydrolysen bringes det partikulære stof over på opløst form og under fermenteringen omdannes/nedbrydes disse opløste stoffer delvist til kortkædede fedtsyrer (VFA; volatile fatty acids) og alkoholer, der efterfølgende fungerer som energikilde for denitrifikationsprocessen.

Forskningen og afhandlingen er inddelt i 3 dele:

1) Karakterisering og estimering af hydrolyse og fermenterings-kapacitet af slam genereret fra to diæter med forskellige proteinkilder; fiskemel (FM) og soyamel (SBM)
2) Optimering af udbytter fra henholdsvis hydrolyse og fermenteringsproces via modifikation af en række procesbetingelser, herunderpH, temperatur, enzym-tilsætning og reaktor-konfiguration (batch fed v*s.* anaerob sequence batch reactor (ASBR)
3) Undersøgelse og praktisk dokumentation på kommercielt dambrug af metode og anvendelighed for anvendelse af slam som intern kulstofkilde til denitrifikation på et relativt ekstensivt drevet moderfiskeanlæg med regnbueørreder

Del 1: Slam resulterende fra anvendelse af to forskellige fodertyper med forskellig proteinkilde (FM og SBM) til juvenile regnbueørreder blev undersøgt for henholdsvis hydrolyse og fermenteringskapacitet.

Herunder blev hydrolyse-udbytte, fermenteringsgrad, kulstof:kvælstof forhold (C:N), frigivelse af næringsstoffer (ammonium og fosfor) undersøgt under 7 dages inkubation, ligesom de dannede lettilgængelig kulstof-kilder blev analyseret og karakteriseret.

Resultaterne afslørede, hvordan sammensætning og fordøjelighed af foderet og næringsstofferne samt fiskenes udnyttelse deraf påvirkede kulstofudbyttet, opløste næringsstoffer og den potentielle udnyttelse af det dannede slam til denitrifikation (Paper I). Øgende proteinindhold i foderet øgede således dannelsen af valin- og eddikesyre mens forøget indhold af NFE (nitrogenfrie ekstraktstoffer; dvs. kulhydrat) i foderet øgede dannelsen af smørsyre og alkohol (ethanol) (Paper II). Inklusion af 10-30% SBM i diæten gav slam med bedre kapacitet for denitrifikation sammenlignet med en standard fiskemels-baseret diæt. Yderligere forøget indhold af SBM op til 40-50% påvirkede imidlertid fermenteringsprocessen negativt og dermed også kapaciteten som intern kulstofkilde til denitrifikation (Manuscript I). Ved at anvende udviklede metoder til at karakterisere hydrolyse- og fermenterings-processerne blev det således demonstreret hvorledes fodersammensætningen påvirker den eventuelle brug af slam som kulstofkilde til end-of-pipe fjernelse af kvælstof, og at dette potentiale kan estimeres og kontrolleres/påvirkes via fodersammensætning.

Del 2: En række eksperimenter blev gennemført med henblik på at optimere hydrolyseudbyttet (degree of solubilization) fra slammet, idet de tidligere resultater havde vist, at kun 21-29% af den totale kulstofmængde i slammet (målt som total-COD) blev bragt over på opløst form under den anaerobe hydrolyseproces.

Betydningen af en række procesbetingelser blev undersøgt herunder pH, temperatur, enzymtilsætning og reaktor-konfiguration og resultaterne viste, at hydrolyse-hastigheden (men ikke udbyttet) kunne forøges ved opretholdelse af konstant pH på 7 samt ved forøget temperatur (40°C). En forøget hastighed kan reducere den nødvendige hydrauliske opholdstid (HRT) og dermed reaktorvolumen. Eftersom alene hastigheden, men ikke udbyttet blev forøget (androg fortsat kun 20-30% af tCOD) kalder resultaterne på fortsatte undersøgelser af hvorledes nedbrydningsprocessen kan forbedres, herunder også hvorvidt hæmmende effekter begrænser processen og udbyttet.

Del 3: Slutteligt blev mulighederne for anvendelse af slam som intern kulstofkilde til denitrifikation på et kommercielt dambrug (moderfiskeanlæg) undersøgt (Manuscript II). Slam fra de normale, daglige rensnings-rutiner (klækkeri/yngel, slamkegler og biofilterskyl) blev anvendt til hydrolyse i to særskilte tanke (SSF; "side stream fermenter") hvori opløste kulstofkilder (dvs. opløst COD og VFA) blev dannet. Disse blev efterfølgende ledt til en denitrifikationsreaktor, hvori den biologiske kvælstoffjernelse skete på basis af det opløste kulstof og tilledt, nitratholdigt vand fra biofilteret. Tre forskellig flows blev undersøgt (6, 18 og 54 m^3/d) og resultaterne viste, at hydrolysen (SSFen) levered en konstant mængde og kvalitet af organiske stoffer til denitrifikationsprocessen. Mængden og "kvaliteten" (dvs. reaktiviteten) af det indsamlede slam på anlægget var dog begrænsende for processen (og dermed kvælstoffjernelsen). Kun 2% udbytte (2% VFA/tCOD) mod optimalt 20-30% gjorde, sammenholdt med slammængden, at den samlede fjernelse blev begrænset.

Selv om reaktiviteten af slammet således var lav, viste beregningerne, at 1 m^3 slam i SSFen var istand til dagligt at fjerne 92.2 g NO_3^--N (plus yderligere 381 g oxygen for gøre opnå anaerobe forhold). Dette betyder, at 27 m^3 slam/dag skulle anvendes til at fjerne de ønskede 2,5 kg NO_3-N/d på det pågældende dambrug med den lave reaktivitet af slammet.

Desuagtet viste forsøget, at den særskilte hydrolyse-proces (SSF) var i stand til at forbedre kvaliteten af slammet for anvendelse som kulstofkilde til end-of-pipe fjernelse af nitratkvælstof. Det er demonstreret, at

processen virker under kommercielle betingelser, og at metoden vil være en reel mulighed/reelt alternativ for kvælstoffjernelse på kommercielle anlæg. Samtidigt understreges vigtigheden af at maksimere slam-mængde og "kvalitet" (reaktivitet; dvs. lav slamalder) med henblik på at få maksimal udbytte af det egenproducerede slam som intern kulstofkilde til kvælstoffjernelse.

Abstract

In Denmark, the implementation of stricter environmental policies has forced the aquaculture sector to improve its practices and water treatment technologies, thus becoming progressively more environmentally sustainable and competitive. Even though the industry has managed to reduce the discharge of organic matter and phosphorous, the major challenge is now the removal of total nitrogen (TN), where only 15-50% can be removed with the best available current technology. From the nitrogenous compounds found in recirculating aquaculture system (RAS) effluent, NO_3^--N constitutes by far the major fraction of TN deriving from biological oxidation of ammonium (NH_4^+), the major N compound excreted by the fish. Therefore, removing the content of NO_3^--N from effluent water is an important step to take. In this respect, heterotrophic denitrification - a biological process where bacteria reduce nitrate (NO_3^-) into gas (N_2) with the use of organic carbon as electron donor – is central. The organic waste produced by the fish in RAS can be used as an internal carbon source for on-farm denitrification. In this way, two waste types (organic waste and nitrate) are treated simultaneously, reducing the associated costs for purchasing external carbon sources and the cost for disposing the organic waste.

Feed is indirectly the major source of the waste produced in RAS, with the digestibility of the feed ingredients along with the macro and micronutrient composition of the feed dictating the amounts and characteristics of the waste produced. Thus, coupling feeding with waste production allows influence on and an estimation of the masses of waste to be treated, including the availability of organic waste that may be used as a resource. The following PhD dissertation focus on enhancing the use of organic waste produced by the fish as an internal carbon source for on-farm denitrification, i.e., transforming the organic waste into a new resource following the "Residual Resource" approach. The research was divided in three parts: 1) characterization and estimation of the hydrolysis and fermentation capacity of the produced organic waste derived from two dietary protein sources, fish meal (FM) and soybean meal (SBM); 2) optimization of the hydrolysis and fermentation yields by modifying different process conditions including pH, temperature, enzymatic addition and reactor configuration (batch fed *versus* anaerobic sequence batch reactor (ASBR)); and finally 3) applying a mass balance approach to evaluate the applicability of using internally produced carbon for denitrification on a low-intensity, Danish rainbow trout (*Oncorhynchus mykiss*) farm.

Part I: Organic waste deriving from two dietary protein sources (FM and SBM) fed to juvenile rainbow trout were characterized in terms of their hydrolysis and fermentation capacity. This entailed examining the hydrolysis yields, degree of fermentation, carbon to nitrogen ratio (C:N), release of nutrients (phosphorous and ammonium) and types of readily available carbon (RAC) compounds produced when incubating the organic waste in laboratory scale batch reactors for 7 days. The results showed how the nutrient composition of feed and the associated digestibility and nutrient utilization by the fish affected carbon yields, nutrient dissolution, and the potential of the carbon compounds produced for denitrification (Paper I). Hence, increasing the protein content in the diet increased the production of valeric and acetic acid, while higher contents of nitrogen free extracts (NFE) in the diet resulted in an increased production of butyric acid and ethanol (Paper II). Inclusions of 10-30% SBM in the diets yielded organic waste with better capacity for denitrification compared to organic waste deriving from a standard FM based diet. However, increasing the inclusion level of SBM up to 40-50% affected the fermentation process negatively, and consequently the capacity of the organic waste as an internal carbon source for denitrification (Manuscript I). By applying methods for individually characterizing the hydrolysis and fermentation process, it was demonstrated how the feed composition affects the potential of using organic waste as an internal carbon source for end-of-pipe removal of N. In effect, the study showed that the waste treatment potential of the

organic waste can be estimated according to the type of feed given to the fish, and/or can be controlled through the type of feed applied.

Part II: A series of optimization experiments were conducted with the aim of increasing the degree of solubilization in organic waste deriving from rainbow trout as the previous results showed that only 21-29% of the total carbon waste, measured as TCOD, was solubilized after hydrolysis under anaerobic conditions. Different process conditions including pH, temperature, enzymatic addition and reactor configuration (ASBR) were evaluated. The results showed that the hydrolysis rate was improved by maintaining a constant pH of 7, or by applying a higher temperature (40^{o}C), reducing the time needed to achieve the same yield as that of the control. Increasing the hydrolysis rate allows for a reduction in the required hydraulic retention time (HRT) and consequently a reduction of reactor volume. Interestingly, none of the evaluated treatments managed to increase the dissolution degree to more than 20-30%. The results warrant a closer look into the nutritional composition and associated degradation properties of the organic waste including potential inhibitory effects that may constrain the dissolution process.

Part III: The applicability of using internal carbon sources for denitrification on a Danish brood stock farm with rainbow trout was evaluated in the last part of the thesis (Manuscript II). Organic waste from the normal, daily cleaning operations (hatchery, sludge cones, and biofilter backwash) was used to feed a side stream fermenter (SSF) producing dissolved carbon (i.e., soluble chemical oxygen demand (sCOD) and volatile fatty acids (VFA)) that were subsequently fed to a denitrification reactor operated at three different flows (6, 18 and 54 m^3/d). The results showed that the SSF delivered a constant quantity and quality of dissolved organic compounds. However, the quality of the collected organic waste limited the performance of the system, resulting in very low fermentation yields (2% VFA/TCOD) compared to what may be obtained under optimal conditions (20-30% VFA/TCOD). Even though the degradability of the recovered organic waste was low, calculations showed that 1 m^3 of the enhanced sludge in the SSF was able to remove 92.2 g NO_3^--N (plus additional 381 g oxygen) on a daily basis. This means, that 27 m^3 of organic waste would be required to remove the 2.5 Kg NO_3-N/d that the farmer has to remove to comply with environmental regulations (removing at the same time 10.3 Kg O_2/d from the incoming water to achieve anoxic conditions in the denitrification reactor). In effect, the SSF enhanced the quality of the organic waste to be used for end-of-pipe removal of N and P, and reduced the amount of organic waste that had to be disposed. Hence, the study demonstrated that the process may be a relevant alternative for end-of-pipe treatment at commercial farms but also that the carbon quantity and quality limited the maximal potential of the process on this specific farm. Improvements in this respect should focus on reducing the masses of oxygen entering the denitrification reactor, adopting a recycling flow within the denitrification reactor, and improving the organic waste collection methods.

Synopsis

1. Background

1.1 Aquaculture environmental challenges

Environmental sustainability has become a key issue in aquaculture particularly regarding source of feed ingredients, alterations of ecosystems and discharge of waste towards water receiving bodies (Martins et al., 2010; Van Rijn, 2013). For an improved sustainability, aquaculture needs a profitable production decoupled from its environmental impact. An increasing number of Danish freshwater farms have converted from traditional open, flow-through systems into Model-Trout-Farms (MTFs) type systems, incorporating a series of cost-efficient water treatment devices and water recycling operations. The technology has allowed these Danish farmers to increase their production capacity within the current environmental regulations (Danish Ministry of Environment, 2012).

The MTFs water treatment systems typically include particle removal devices (for example sludge cones and drum filters), and aerobic, nitrifying biological filters for converting NH_4^+ to NO_3^-, allowing for a water recirculation intensity of min. 95% (MTF type III). The farm effluent is treated in constructed wetlands before finally being discharged into receiving water bodies. A constructed wetland can remove up to 75% of biochemical oxygen demand (BOD), 60% of total phosphorous (TP) and, at best, 50% of total nitrogen (TN), this last parameter being one of the main factors limiting the Danish (and European) industry from increasing its production (Jokumsen and Svendsen, 2010; Dalsgaard et al., 2013). To overcome this challenge, efforts are concentrated on developing cost effective technologies for removing TN from the RAS discharge water, with special emphasis on NO_3^--N, which constitutes more than 80% of TN (Timmons et al., 2008; Diaz et al., 2012).

Denitrification is a microbial process by which nitrate (NO_3^-) is reduced into atmospheric nitrogen (N_2) with the use of organic matter as an electron donor (Eq. 1).

$$5CH_3COO^- + 8NO_3^- + 3H^+ \rightarrow 10HCO_3 + 4N_2(g) + 4H_2O \quad \textbf{(Eq. 1)}$$

The efficiency by which nitrate is reduced closely depends on the availability of easy biodegradable carbon sources. The addition of methanol and acetic acid is a frequent commercial practice to boost the process, showing good results in terms of stability and controllability (Hamlin et al., 2008; Henze et al., 2002; Ucisik and Henze, 2008). However, the addition of external carbon sources increases the process operational costs and the sludge production. Organic waste produced by the fish in RAS has shown to have potential for serving as an internal carbon source for denitrification (Jewel and Cummings, 1990; van Rijn et al., 2006; Tal et al., 2009; Suhr et al., 2014), but no further research fully explores its potential.

1.2 Feed, an indirectly main source of waste in aquaculture

Feed is indirectly the major source of waste produced in aquaculture systems, the composition and formulation influences the physical and chemical properties of the waste and affects the organic matter, nitrogen and phosphorous output masses (Cho et al., 1994; Nijhof, 1994; Talbot and Hole, 1994; Timmons et al., 2009; Dalsgaard and Pedersen, 2011). It has previously been reported that in diets based on fish meal a reduction in the digestible protein to digestible energy ratio (DP:DE) can influence the nitrogen waste output (Cho and Bureau, 2001; Green and Hardy, 2008). In the case of diets from vegetable proteins, efforts have majorly focused on fish digestibility (Brinker and Reiter, 2010; Pratoomyot et al., 2010; Collins et al., 2013)

while not much information exists about the influence of the solid and dissolved waste produced (Amirkolaie, 2005).

Information on diet formulations, related apparent nutrient digestibility and nutrient utilization (in terms of feed conversion ratios) can be used to predict specific effluent types and masses, and by this improve biological nutrient removal (Dalsgaard and Pedersen, 2011). This would allow the industry to minimize the environmental impact of the effluents and promote increasingly more sustainable practices.

1.3 Denitrification as end-of-pipe treatment in the aquaculture industry

Denitrification as an end-of-pipe treatment technology in aquaculture gains increasingly more attention as reducing the discharge of N has become a necessity for complying with environmental regulations (Hamlin et al., 2008; Suhr et al., 2012; van Rijn, 2013). Bacterial mediated denitrification is frequently used in wastewater treatment where it has proven to be a more cost effective solution than ammonia stripping, breakpoint chlorination, and ion exchange (Metcalf and Eddy, 2004). Similarly, it has turned out to be one of the most promising and versatile technologies to remove nitrate from aquaculture water. Biological denitrification as end-of-pipe treatment in aquaculture has been carried out using autotrophic bacteria (Saliling et al., 2007; Christianson et al., 2015; von Ahnen et al., 2016) and heterotrophic bacteria (Klas et al., 2006; Tal et al., 2009; Suhr et al., 2014). Most denitrifying organisms are facultative anaerobe heterotrophs, i.e., they have the ability to switch their metabolism from using oxygen as an electron acceptor to using nitrate instead. Under aerobic conditions the bacteria will use oxygen rather than nitrate as oxygen is energetically favored over nitrate (Henze et al., 2002). These circumstances have proven to negatively affect the efficiency of denitrifying reactors in aquaculture as the water to be treated usually has high oxygen concentrations (van Rijn and Rivera, 1990; Klas et al., 2006). Therefore, to pursue efficient denitrification there must be enough carbon to accomplish three main processes: 1) respiration with oxygen to achieve anoxic conditions 2) reduction of NO_3^--N to N_2; and 3) bacterial growth; (Henze et al., 2008). In this sense, the relation between the mass of carbon and nitrate available for the denitrification process are key characteristics, and is usually expressed in terms of the C:N ratio.

1.4 Carbon to NO_3^- ratio required for achieving denitrification in RAS

If few electron donors (expressed in terms of the Carbon Oxygen Demand, COD) are provided for the bacteria, i.e., if the C:N ratio is too low, complete denitrification cannot be achieved. A low C:N ratio not only affects the capacity for removing nitrate, but can also lead to an increment in intermediate products such as nitrite, which may cause methemoglobinemia in terrestrial organisms and fish (Wolf and Wasserman, 1972; Timmons et al., 2009), or the release of di-nitrogen oxide which is a 300-fold stronger greenhouse gas than CO_2 (Ni et al., 2011). In the opposite case, i.e., where an excess of available carbon is present, bacteria may perform dissimilatory nitrate reduction to ammonium (Tiedje, 1992). This is an unwanted process where nitrate is not removed from water but rather converted "back" to its original aquaculture form (ammonium). For these reasons, one of the main challenges of this technology is to calculate the available mass of COD with respect to the available mass of electron acceptors (NO_3^-) taking into account the biological kinetics of bacteria and the system operating parameters (aerobic/anoxic conditions, recycle ratios and system configuration) (Metcalf and Eddy, 2004; Henze et al., 2008).

Different C:N ratios have been reported in aquaculture. Klas et al. (2006) achieved maximum nitrate removal rates of 590 mg N/L reactor/d at a C:N ratio of 4.5-4.9 using a 15 L single-sludge denitrification reactor operated at a solid retention time (SRT) of 4 days. In a 5.5 m^3 up-flow single-sludge denitrification setup with a C:N ratio of 6.9 and a HRT of 98 min, Suhr et al. (2012) reached a maximal denitrification rate of 125

g NO_3^--N/m^3 reactor/d. Huliñir et al. (2012) used salmon (*Salmo salar*) organic waste as carbon source in 1 L anoxic batch reactors and found that nitrate as well as nitrite were completely removed at a C:N ratio between 7.8-63 (Total organic carbon (TOC:NO_3^--N)) in a time interval of 10-14 h, during which maximum nitrite concentrations ranged between 17.6 – 106 mg NO_2^--N/L, increasing as the C:N ratio decreased. At a C:N ratio of 2, a maximum concentration of 591 mg NO_2^--N/L was found, were 98.3% of the nitrite concentration was reduced after 36 h. van Rijn and Rivera (1990) achieved removal rates between 55 – 352 mg NO_3^--N/m^3/min using 131.5 L fluidized bed columns, finding high variations in nitrate removal with concomitant nitrate accumulation. Based on this it was concluded that nitrite accumulation may happen if organic carbon from the culture unit (internal carbon sources) is used as carbon source for denitrification.

Even though the C:N ratio for denitrification in aquaculture has been examined, there is still a need to develop a more complete understanding about the dynamics of the different nitrogen states, available nutrients, bacterial communities and carbon sources (internal or external) (Klas et al., 2006; van Rijn et al., 2006). Suboptimal environmental conditions provided to the microorganisms, as well as a bad system design, will lead to inefficient results and utilization of internal carbon sources, and will increase the use of resources as for example external carbon sources (Henze et al., 2002; Suheyl and Henze, 2008).

1.5 Endogenous and exogenous carbon sources in RAS

Denitrifying bacteria can use a wide spectrum of carbon sources, classified as external (often commercially obtained) or internal (produced within the system) sources (Henze et al., 2008). Applying external carbon sources for denitrification has been evaluated in RAS with the objective of increasing the water recirculating intensity of the system or improving temperature control. Otte and Rosenthal (1979) used glucose and methanol as carbon sources in a denitrification reactor for an European eel (*Anguilla anguilla*) culture achieving around 50% removal of nitrate. Suzuki et al. (2003) used methanol for a zero discharge eel culture system, reducing 90% of the nitrate accumulated in the rearing tank. Hamlin et al. (2008) evaluated four different carbon sources (methanol, acetic acid, molasses and a hydrolyzed starch) reaching denitrification rates of 670-800 g NO_3^--N/m^3 media/d, and showing effective removal of nitrate to near zero concentrations. Other alternative carbon sources have also been evaluated for heterotrophic denitrification. Boley et al. (2000), using biodegradable polymer pellets in fish aquaria, achieved volumetric removal rates between 13 -16 mg NO_3^--N/L/h with effluent nitrate levels below the detection limit (<0.23 mg NO_3^--N/L). Similarly, Gutierrez et al. (2011) used polyhydroxybutyrate as a carbon media and found high removal rates in the order of 2.5 Kg NO_3^--N/m^3 media/d. Adding external carbon sources to denitrification systems in aquaculture has thus proven to be an effective solution for controlling nitrate levels. However, this practice increases the operational costs and production of sludge, the later eventually requiring further treatment. It may thus be estimated that if methanol is used for reducing the NO_3^- deriving from the production of 1 Kg of rainbow trout, around 5-10% of the total production costs would be spent on buying methanol, which makes it a less attractive solution, especially if applied for end-of-pipe treatment on low-medium priced fish species.

To save operational costs in RAS, internal carbon sources originating from fish faeces can be used for end-of-pipe denitrification, a configuration that allows the simultaneous removal of organic matter and nitrate (Jewell et al. 1990; Arbiv and van Rijn, 1995; Tal et al., 2009; Suhr et al., 2014). One of the main discussions regarding the feasibility, and potential limiting factors, of using internal carbon sources for denitrification relates to the availability of organic waste produced and captured in the system. Few data exists on the chemical composition, electron donating properties and biodegradability characteristics of the recoverable organic waste generated in RAS (Klas et al., 2006; van Rijn et al., 2006; Conroy and Couturier,

2010). Quantifying the masses and types of the organic matter is of major importance for predicting the capacity of the internal carbon sources for on-farm denitrification. Furthermore, as the feed composition, and consequently the composition of faecal waste feeding into the waste treatment system (N, P and organic waste) are relatively constant (Dalsgaard and Pedersen, 2011), there is a high potential for predicting and estimating the capacity for biological waste treatment.

1.6 Hydrolysis and fermentation of discharged RAS organic waste

Contrary to municipal raw sewage, the majority of the organic waste in RAS is not present as dissolved matter readily available for the bacterial consumption, but rather as particulate matter that needs to undergo hydrolysis prior to bacterial degradation. To optimize the applicability of the internal carbon sources, the conversion of settleable faecal solids (SFS) to readily available carbon sources (RAC), as for example volatile fatty acids (VFAs), can be achieved through anaerobic digestion as initially demonstrated by anaerobically degrading fish feed (Arbiv and van Rijn, 1995; van Rijn et al., 1995). In continuation of this approach, the production of VFAs from aquaculture waste streams under laboratory conditions has been described, and yields between 0.13 and 0.21 g VFA/g total volatile solids (TVS) have been reported (Conroy and Courier, 2010; Suhr et al., 2014; Paper I). The effect of different types of VFA, produced from the organic waste, on the denitrification process in aquaculture has, however, as far as known not been studied. Coming close, Aboutboul et al. (1995) quantified the effect of artificially added acetate, propionate and butyrate, as well as a mixture of these three VFAs, on the denitrification process and found that propionate resulted in the highest nitrate removal rates. In industrial and municipal wastewater treatment, Fass et al. (1994) in an activated sludge system found that acetate, butyrate, valerate by themselves or in a mixture including propionate resulted in the same denitrification rate (19 mg NO_3^--N/g suspended solids (SS)/h) and carbon consumption (60 mg C/g SS/h), whereas propionate used alone was almost not metabolized. Elefsioniotis and Li (2006) found that acetic acid was consumed at a higher rate than propionic acid, attributing it to a simpler metabolic pathway of degradation. They furthermore discussed that a temperature between 10-20^{0}C compared to 20-30^{0}C exerted a higher effect on the specific denitrification rates and carbon consumption rates. Her and Huang (1995), using a batch reactor, reported how the chemical structure and molecular weight of the carbon source used correlates well with denitrification efficiency. Hence, lower efficiencies were obtained when using benzoic acid (an aromatic carbon source) compared to using methanol, acetic acid or glucose (non-aromatic carbon sources). Furthermore, they found that an excess of methanol and benzoic acid, with respect to the C:N ratio, inhibited denitrification. Lee and Welander (1996) evaluated four carbon sources including acetic acid, crude syrup, hydrolyzed starch and methanol, and found that all four carbon types were suitable for denitrification but that they had a significant influence on denitrification yields, denitrification rates, sludge production and bacterial microflora. Acetic acid and methanol resulted in higher denitrification rates and yields and less production of sludge as compared to crude syrup and hydrolyzed starch.

1.7 Overall objectives of The PhD study

The main objective of this PhD thesis was to optimize the hydrolysis and fermentation process of organic waste produced by rainbow trout with the aim of improving end-of-pipe denitrification in commercial aquaculture. Based on the fact that fish feed is the main, indirect source of waste in aquaculture, the project started out by characterizing the hydrolysis and fermentation process of the waste as well as the types of simple carbons compounds produced from different dietary compositions and protein sources. The following were the objectives for **Part I** of the study:

a) Develop indicators for describing the hydrolysis, fermentation and nutrient dissolution processes in order to compare dietary treatments.

b) Characterize organic waste masses and the potential for producing volatile fatty acids.

c) Estimate and compare the potential for pursuing denitrification using organic waste deriving from either fish fed fish meal based or soybean based diets.

Subsequently, the capacity for optimizing the hydrolysis and fermentation yields of the organic waste was evaluated under different process conditions with the following objective for **Part II** of the study:

d) Increase the degree of solubilization sCOD/TCOD (g/g).

Finally, in **Part III** of the study a side stream fermenter (SSF) was installed at a Danish brood stock farm to evaluate the feasibility of using internal carbon sources under commercial scale conditions for removing nitrate from the effluent stream with the following objective:

e) Evaluate the applicability of a SSF for producing internal carbon sources for denitrification on a Danish trout farm.

The research aimed at resolving how organic waste produced in aquaculture can be considered as a residual resource, reducing the needs and costs for purchasing external carbon to remove nitrate via heterotrophic denitrification.

2. Part I: Characterizing and describing the hydrolysis and fermentation processes of solid waste deriving from two dietary protein sources, fish meal and soybean meal

2.1 Indicators applied for evaluating the hydrolysis and fermentation processes

One of the concepts pursued in this thesis was to evaluate the possibility of predicting the potential for performing denitrification using endogenous carbon sources produced from different dietary treatments. In order to accomplish this objective the first step was to characterize the properties of the SFS, deriving from each dietary treatment, and normalize it to the amount of feed consumed by the fish (Table 1).

Table 1. Characteristics (day 0) of settable faecal solids (SFS) produced by fish fed diets with increasing ratios (15 to 23) of protein:energy (P:E) and posteriorly used in the hydrolysis/fermentation batch study (mean ± SD, n=3). Data are expressed as masses produced/feed consumed. The SFS were collected during four consecutive days (4x24 h) and pooled prior to the incubation study[1]. Data extracted from Letelier-Gordo et al. (2015).

Dietary treatments	P:E_15	P:E_17	P:E_19	P:E_21	PE:_23
TS (g/g)[2]	$0.19^{a} \pm 0.01$	$0.17^{a} \pm 0.01$	$0.17^{a} \pm 0.02$	$0.18^{a} \pm 0.01$	$0.20^{a} \pm 0.01$
TVS (g/g)[3]	$0.14^{c} \pm 0.01$	$0.11^{ab} \pm 0.01$	$0.10^{ab} \pm 0.01$	$0.10^{ab} \pm 0.01$	$0.12^{ac} \pm 0.00$
TKN (mgN/g)[4]	$5.8^{d} \pm 0.4$	$6.5^{bd} \pm 0.4$	$7.1^{bc} \pm 0.8$	$8.1^{ac} \pm 0.4$	$8.6^{a} \pm 0.4$
Protein (mg/g)[5]	$36.0^{d} \pm 2.2$	$40.8^{bd} \pm 4.7$	$44.6^{bc} \pm 4.7$	$50.6^{ac} \pm 2.2$	$53.7^{a} \pm 2.4$
Lipid (mg/g)	$29.2^{a} \pm 3.3$	$21.0^{a} \pm 4.8$	$21.8^{a} \pm 5.6$	$24.0^{a} \pm 6.1$	$28.1^{a} \pm 2.9$
NFE (mg/g)[6]	$100.7^{b} \pm 1.3$	84.1 ± 6.4^{a}	73.0 ± 7.7^{a}	71.8 ± 2.8^{a}	84.3 ± 4.2^{a}
TP (mg/g)[7]	$7.9^{a} \pm 0.9$	$11.2^{a} \pm 2.1$	$9.6^{a} \pm 1.1$	$9.1^{a} \pm 3.1$	$9.4^{a} \pm 2.5$
Ash (mg/g)	49.9 ± 4.4^{a}	60.7 ± 4.2^{ac}	66.6 ± 5.3^{ce}	74.0 ± 2.9^{bde}	76.0 ± 2.9^{bde}

[1] Values within rows not sharing a common superscript letter were significantly different (Tukey-Kramer, $P<0.05$).
[2] TS: Total solids at day 0
[3]TVS: Total volatile solids at day 0.
[4]TKN: Total Kjeldahl nitrogen
[5]Protein was derived as total Kjeldahl nitrogen (TKN) multiplied by 6.25.
[6]NFE: Nitrogen free extract calculated as NFE = TS – protein – lipid – ash.
[7]TP: Total phosphorous

With the developed waste coefficients derived from the proximal composition of the SFS, the different masses of protein, lipids, carbon, phosphorous, carbohydrates (nitrogen free extracts) produced per mass of feed consumed can be estimated (Eq. 2). In this way, the available mass of carbon (TS, TCOD) and the required amount of N (TKN) to be removed via denitrification per feed consumed can be estimated.

$$\textbf{Waste coefficient} = \frac{\text{waste produced as TS;TCOD; Protein; Lipid; TP}}{\text{feed consumed}} \text{ (g/g)} \qquad \textbf{(Eq. 2)}$$

Very few studies have reported the hydrolysis capacity of fish sludge. In addition, different approaches and nomenclatures have been used to describe the hydrolysis and fermentation processes, making a comparison of results between studies difficult. Van Rijn et al. (1995) described the release of VFAs from anaerobically degrading fish feed, and expressed the results as VFAs (mg/L) accumulated through time. Conroy and Couturier (2010) and Suhr et al. (2014) expressed the hydrolysis/fermentation process by normalizing the accumulated concentration of soluble COD and VFAs to the total volatile solid (TVS) concentration of the sludge measured at day 0 (sCOD/TVS0; VFA/TVS0). In the latter cases, the production of VFAs was

reported as one complete process (Conroy and Couturier, 2010; Suhr et al., 2014), whereas it in strict terms covers for two coupled processes, namely solubilization and fermentation.

In the current study, the solubilization and fermentation processes were described separately in order to better compare the dynamics between the different dietary treatments. The degree of solubilization or the capacity of bacteria to produce soluble compounds (compounds less than 0.2 μm) from SFS under anaerobic conditions is expressed as sCOD/TCOD (eq. 3). Subsequently, the capacity of bacteria to produce readily available carbon sources (VFAs) from the already solubilized organic matter is defined as the degree of fermentation and is expressed as VFAs/sCOD (eq. 4). In a similar manner and to quantify the degree to which nutrients such as ammonia and orthophosphate were released to the bulk phase due to the hydrolysis process, the expression NH_4^+/TKN (eq. 5) was applied for ammonia dissolution while, analogously, the expression PO_4^{3-}/TP (eq. 6) was used for orthophosphate dissolution.

The C:N ratios, or the potential capacity of the SFS to sustain denitrification were evaluated using two indicators including the C:N obtained (VFA/TKN) (eq. 7) after 7 days of hydrolysis/fermentation and the C:N potential (TCOD/TKN) (eq. 8).

$$\textbf{Degree of solubilization} = \frac{\text{sCOD}}{\text{TCOD}}\ (\text{g/g}) \qquad \textbf{(Eq. 3)}$$

$$\textbf{Degree of fermentation} = \frac{\text{VFA\_COD}}{\text{sCOD}}\ (\text{g/g}) \qquad \textbf{(Eq. 4)}$$

$$\textbf{Nitrogen solubilization} = \frac{\text{TAN}}{\text{TKN}}\ (\text{g/g}) \qquad \textbf{(Eq. 5)}$$

$$\textbf{Phosphorous solubilization} = \frac{PO_4^{3-}}{\text{TP}}\ (\text{g/g}) \qquad \textbf{(Eq. 6)}$$

$$\textbf{C:N obtained} = \frac{\text{VFA\_COD}}{\text{TKN}}\ (\text{g/g}) \qquad \textbf{(Eq. 7)}$$

$$\textbf{C:N potential} = \frac{\text{TCOD}}{\text{TKN}}\ (\text{g/g}) \qquad \textbf{(Eq. 8)}$$

For calculation purposes, VFA values were expressed on a COD basis according to Henze et al., 2008 (Table 2).

Table 2. Stoichiometric values of COD for different pure organic compounds (Henze et al., 2008)

Name	Formula	COD
Formic acid	CH_2O_2	0.35
Acetic acid	$C_2H_4O_2$	1.07
Propionic acid	$C_3H_6O_2$	1.51
Butyric acid	$C_4H_8O_2$	1.82
Valeric acid	$C_5H_{10}O_2$	2.04
Ethanol	C_2H_6O	2.09
Methanol	CH_3OH	1.50

2.2 Indicators applied for estimating carbon production

With the developed indicators (eq 3 and 4) and the waste mass characterization (Table 1) the amount of VFAs that can be produced can be estimated. Values can be expressed as VFA_COD produced/ mass feed consumed (Eq. 9) or VFA_COD produced/mass of fish produced (Eq. 10).

$$\frac{\textbf{VFA\_COD produced}}{\textbf{Feed consumed}} = \frac{\text{TCOD produced}}{\text{Feed consumed}} * \frac{\text{sCOD}}{\text{TCOD}} * \frac{\text{VFA}}{\text{sCOD}} \qquad \textbf{(Eq. 9)}$$

$$\frac{\textbf{VFA\_COD produced}}{\textbf{mass fish produced}} = \text{FCR} * \frac{\text{TCOD produced}}{\text{Feed consumed}} * \frac{\text{sCOD}}{\text{TCOD}} * \frac{\text{VFA}}{\text{sCOD}} \qquad \textbf{(Eq. 10)}$$

where $\text{FCR} = \frac{\text{Feed consumed}}{\text{mass fish produced}}$

The following step was carried out to characterize, describe and estimate the capacity of using organic waste for denitrification deriving from rainbow trout fed five, iso-energetic, fish meal based diets with increasing ratios of protein:energy.

2.3 Reducing the dietary protein:energy (P:E) ratio affects the solubilization and fermentation of rainbow trout (*Oncorhynchus mykiss*) faeces (Paper I)

Experimental design

A randomized, single-factor experiment was performed using five fish meal isoenergetic experimental diets (P:E 15, 17, 19, 21, 23) with different levels of protein:energy, and three replicate tanks (n=3) for each diet. Ingredients and analyzed proximate composition of the diets are shown in Table 3. The fish were maintained in 15 separate, flow-through tanks in a nutrient mass balance system (NMBS) as described in Dalsgaard and Pedersen (2011) (Figure 1).

Table 3. Ingredients used and analyzed gross composition of the five experimental diets. Data extracted from Letelier-Gordo et al. (2015).

Ingredients (%)	**P:E 15**	**P:E 17**	**P:E 19**	**P:E 21**	**P:E 23**
Fish meal[1]	42.9	50.8	58.7	66.5	74.4
Wheat	37.3	30.4	23.6	16.7	9.8
Fish oil	21.2	19.9	18.6	17.3	16.0
Vitamins & minerals[2]	0.30	0.30	0.30	0.30	0.30
Proximate composition (%)[3]					
Dry Matter	93.4	93.5	93.7	94.8	96.6
Protein	32.7	37.2	42.5	46.9	50.2
Lipid	27.2	26.4	25.9	25.0	24.5
Ash	7.55	8.71	9.86	11.0	11.2
Phosphorous	1.3	1.5	1.7	1.9	1.9
NFE (nitrogen free extracts)[4]	24.6	19.7	13.7	10.0	8.8
Gross energy (Kj/g)[5]	22.5	22.3	22.1	22.0	22.4

[1] SA 68 superprime Perú, South America (68% protein). [2] Premix Dk 3. [3] Proximate composition analyzed as described in Dalsgaard and Pedersen (2011). [4] NFE calculated as: dry matter – protein –lipid – ash. [5] Gross energy measured using a bomb calorimeter (IKA-Calorimeter C7000, IKA Analystechnik, Heitersheim, Germany).

Fig. 1. Nutrient mass balance system (NMBS) as described in Dalsgaard and Pedersen (2011) and used in the experimental trials.

The produced settable faecal solids (SFS) from four consecutive days were collected and pooled prior to incubation. The SFS were stored at 0^0C to minimize potential degradation. Posteriorly the collected SFS were transferred into 15, 1L anoxic/anaerobic batch reactors maintained at 20 ± 2^0C with continuous magnetic stirring at 200 rpm (Figure 2). A summary of the characteristics of the SFS examined in the hydrolysis/fermentation trial (i.e. at day 0) is presented in Table 4. Daily samples from the batch reactors were obtained for 7 successive days and analyzed for total ammonia nitrogen (TAN), phosphorus expressed as orthophosphate (PO_4^{3-}-P), VFA and sCOD.

Fig. 2. Anoxic/anaerobic 1 L batch reactors used in the experiment.

Table 4. Characteristics (day 0) of settable faecal solids (SFS) produced from the different diets and posteriorly used in the hydrolysis/fermentation batch study (mean ± SD, n=3). Data are expressed as masses produced/feed consumed, and are based on daily sampling and subsequent pooling for four consecutive days[1]. Data extracted from Letelier-Gordo et al. (2015).

Diet	P:E_15	P:E_17	P:E_19	P:E_21	PE:_23
TS (g/g)	$0.19^{a} \pm 0.01$	$0.17^{a} \pm 0.01$	$0.17^{a} \pm 0.02$	$0.18^{a} \pm 0.01$	$0.20^{a} \pm 0.01$
TVS (g/g)	$0.14^{c} \pm 0.01$	$0.11^{ab} \pm 0.01$	$0.10^{ab} \pm 0.01$	$0.10^{ab} \pm 0.01$	$0.12^{ac} \pm 0.00$
TKN (mgN/g)	$5.8^{d} \pm 0.4$	$6.5^{bd} \pm 0.4$	$7.1^{bc} \pm 0.8$	$8.1^{ac} \pm 0.4$	$8.6^{a} \pm 0.4$
Protein (mg/g)[2]	$36.0^{d} \pm 2.2$	$40.8^{bd} \pm 4.7$	$44.6^{bc} \pm 4.7$	$50.6^{ac} \pm 2.2$	$53.7^{a} \pm 2.4$
Lipid (mg/g)	$29.2^{a} \pm 3.3$	$21.0^{a} \pm 4.8$	$21.8^{a} \pm 5.6$	$24.0^{a} \pm 6.1$	$28.1^{a} \pm 2.9$
NFE (mg/g)[3]	$100.7^{b} \pm 1.3$	84.1 ± 6.4^{a}	73.0 ± 7.7^{a}	71.8 ± 2.8^{a}	84.3 ± 4.2^{a}
TP (mg/g)	$7.9^{a} \pm 0.9$	$11.2^{a} \pm 2.1$	$9.6^{a} \pm 1.1$	$9.1^{a} \pm 3.1$	$9.4^{a} \pm 2.5$
Ash (mg/g)	49.9 ± 4.4^{a}	60.7 ± 4.2^{ac}	66.6 ± 5.3^{ce}	74.0 ± 2.9^{bde}	76.0 ± 2.9^{bde}
Fish performance					
SGR[4]	$2.10^{a} \pm 0.04$	$2.27^{ab} \pm 0.03$	$2.30^{abc} \pm 0.09$	$2.52^{bc} \pm 0.12$	$2.55^{c} \pm 0.06$
FCR[5]	$0.82^{a} \pm 0.02$	$0.75^{ab} \pm 0.01$	$0.74^{b} \pm 0.03$	$0.67^{c} \pm 0.03$	$0.66^{c} \pm 0.02$

[1]Values within rows not sharing a common superscript letter were significantly different (Tukey-Kramer, $P<0.05$).
[2]Protein was derived from TKN by multiplying by 6.25.
[3]NFE was calculated as NFE = TS – protein – lipid – ash.
[4]SGR: Specific growth rate calculated as $Ln(W(t_i)/W(t_0))/(t_i-t_0) \times 100$, $W(t_i)$ and $W(t_0)$ being the biomass at the end (t_i) and start (t_0) of the growth evaluation period (9 days).
[5] FCR: feed conversion ratio calculated as feed consumed (t_i-t_0)/biomass gain (t_i-t_0).

2.3.1 Degree of solubilization and fermentation of sludge from fish meal based dietary treatment groups

Applying the above developed indicators (eq 3 and 4), the results showed how the degree of solubilization of the organic waste is affected by the dietary treatment. Organic waste deriving from the two diets with the lowest protein content (P:E_15 and 17) reached significantly higher yields measured as sCOD/TCOD compared to the treatment group fed the diet with a P:E ratio of 21 (0.30-0.29 versus 0.24 g sCOD/g TCOD) (Figure 3a). Inversely to the results obtained for the degree of solubilization, the organic waste deriving from the lowest P:E ratio diet (i.e. P:E_15) showed the lowest degree of fermentation measured as VFA/sCOD compared to the organic waste deriving from the other four diets with higher P:E ratios (0.36 versus 0.51-0.56 g VFA/g sCOD, respectively) (Figure 3b).

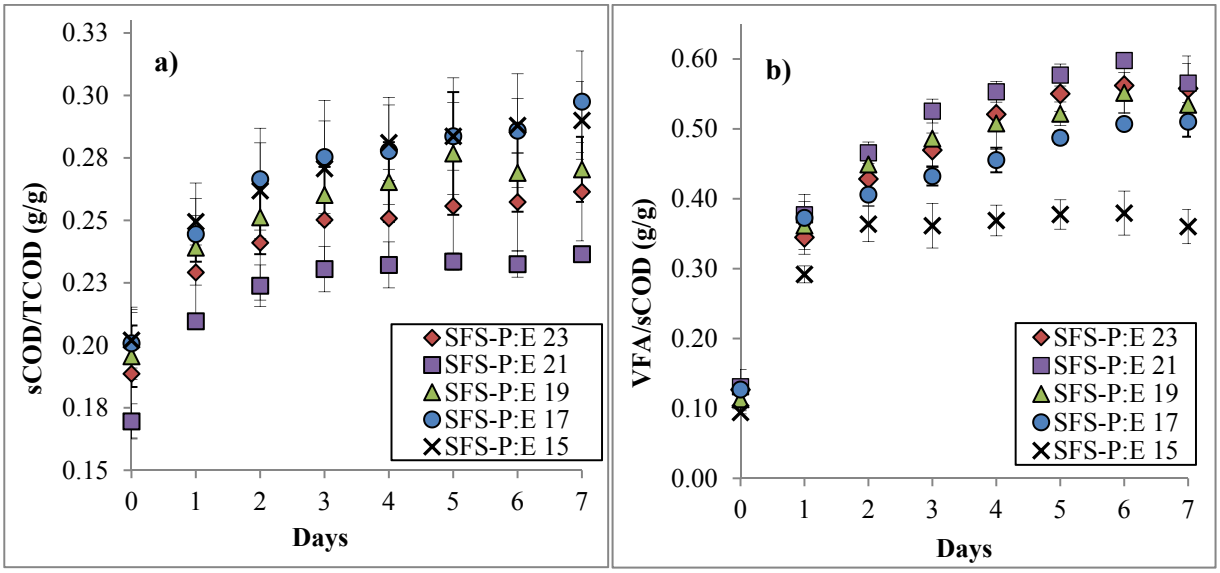


Fig. 3. (**a**) Cumulative degree of solubilization (sCOD/TCOD) during 7 days of incubation of sludge (SFS) deriving from rainbow trout fed diets with increasing ratios of protein:energy (P:E; mean ± SD, n=3). No significant difference between treatment groups was found at day 0. SFS-P:E 15 and SFS-P:E 17 were significant higher compared to SFS-P:E 21 at day 7. **(b)** Cumulative degree of fermentation (VFA/sCOD) for each treatment groups throughout the 7 days of incubation (mean ± SD, n=3). No significant difference between feed types was found at day 0. SFS-P:E 15 was significantly lower than the rest of the SFS at day 7. Data extracted from Letelier-Gordo et al. (2015).

The obtained degree of fermentation (36-57%; Figure 3b) during the 7 days of incubation was generally well below values reported for wastewater treatment plants (83-99%; Cokgor et al., 2008; Suheyl and Henze, 2008), and also aquaculture (74-76%; Suhr et al., 2014). Furthermore, in dietary treatments P:E_17-23 46-49% of the solubilized COD was not converted to VFAs, a situation even more pronounced for the P:E_15 treatment group where up to 64% of sCOD was not converted. In other words, lower P:E ratio diets lead to a significantly higher degree of solubilization of the sludge but a lower degree of fermentation. A potential explanation for this might be that the bacteria consortia involved, which mainly derived from the intestine of the fish, possessed a limited fermentative activity towards a substrate that had also previously not been digested or absorbed "within" the fish. It may also be noticed, that an accumulation of VFAs in the reactor caused a drop in pH (Figure 4), which could have affected the activity of the bacteria. Hidalgo et al. (1998) reported that at pH 6.0 the proteolytic activity in rainbow trout intestines was reduced by approximately 30% of its full activity (at pH 8.5). Furthermore, the capacity of acidogenic/fermentative bacteria to use all the available sCOD for producing VFAs might have been reduced, since an accumulation of fermentation end products, including primarily organic acids (i.e. VFAs), occurred, potentially creating a feedback inhibition as described by Gerardi (2006).

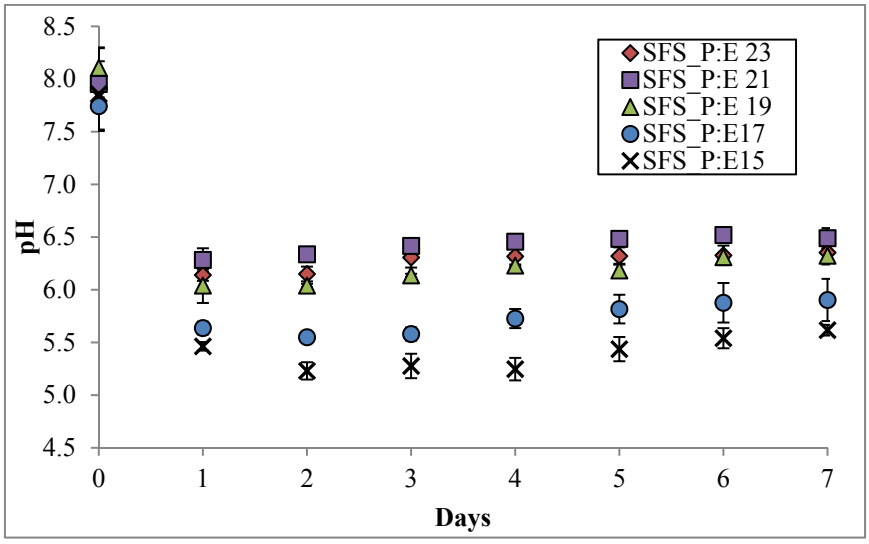


Fig. 4. pH values measured throughout the incubation period (mean ± SD, n=3). No significant difference between dietary treatments was found at day 0, whereas significant difference between SFS-P:E 15 and SFS-P:E 17 and the rest of the evaluated SFS was found at day 7. Significant differences were found at day 1 between SFS_P:E15 and P:E 17 as compared to SFS_ P:E 19, P:E 21 and P:E 23. Data extracted from Letelier-Gordo et al. (2015).

2.3.2 Nutrient dissolution between fish meal dietary treatments

A significantly lower dissolution of total ammonia nitrogen (TAN) was obtained in the lower P:E diet compared to the three highest protein diets (i.e. P:E_15 vs P:E 19-23): 0.08 vs 0.26-0.34 g TAN/g TKN (Figure 5a). In contrast, the two lowest protein diets (i.e. P:E_15 and 17) resulted in the highest solubilization of phosphorous (0.15 and 0.08 g/g PO_4^{3-}P/TP, respectively), which was probably due to a lower pH obtained during the fermentation process (Figure 5b).

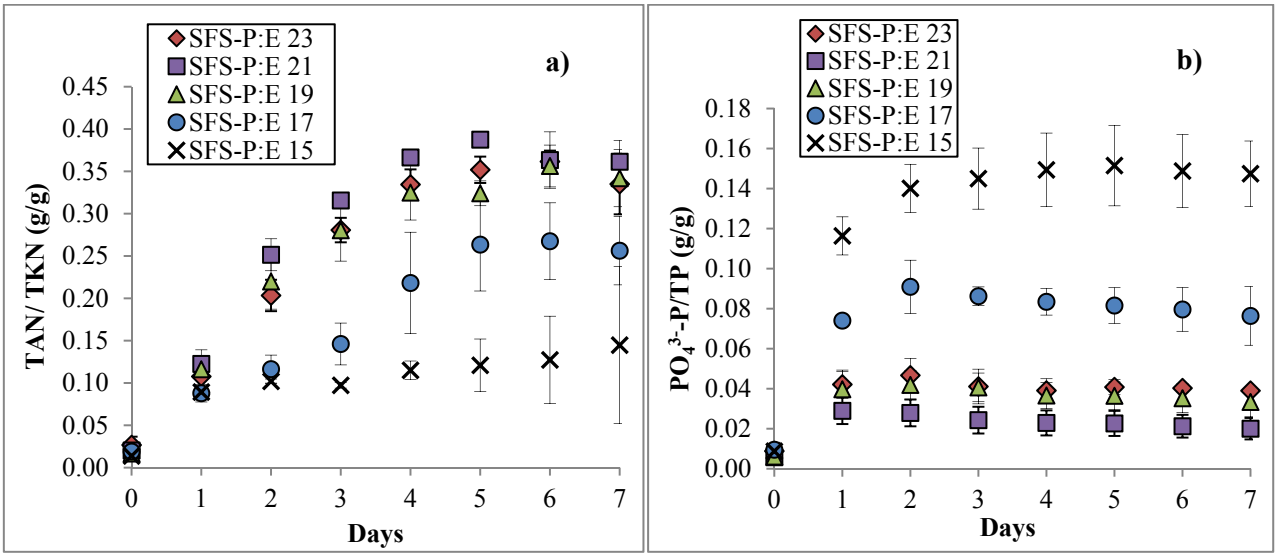


Fig. 5. (**a**) Dissolution of TAN shown as g TAN/g TKN during incubation of sludge from each feed type (mean ± SD, n=3). No significant differences were found between treatment groups at day 0, while SFS-P:E 19, SFS-P:E 21, and SFS-P:E 23 were significantly higher than SFS-P:E 15 from day 2 onwards. **(b)** Dissolution of phosphorus expressed as PO_4^{3}-P/TP measured during incubation of each treatment group (mean ± SD, n=3). No significant differences were found between feed types at day 0, while SFS-P:E 15 and SFS-P:E 17 were significantly higher than the other treatment groups from day 1 onwards. Data extracted from Letelier-Gordo et al. (2015).

In general, through the developed indicators for the hydrolysis and fermentation processes, the study demonstrated how the composition of the feed and specifically the different levels of protein in the diet affected the production of soluble carbon as well as the associated nutrient dissolutions (TAN and $PO_4^{3-}P$).

The solubilization of the organic waste only ranged between 20-30% (i.e., 0.20 – 0.30 g sCDO/g TCOD), and the next step was therefore to explore if and how the yields could be improved, i.e., resolving the full potential of the SFS as an endogenous carbon sources (Part II: Optimization). Moreover, the degree of fermentation (VFA/sCOD) found was lower than previously reported in aquaculture waste and wastewater treatments. To resolve why this was the case, the next part of the study explored whether there was a feedback inhibition effect hampering the fermentation process or if the method for quantifying the carbon products was not the most appropriate, since carbon products other than VFAs (e.g., alcohols) are known to be produced in the fermentation of the organic waste.

2.4 The composition of readily available carbon produced by fermentation of fish faeces is affected by the dietary composition (Paper II and Manuscript I).

The type of carbon compounds applied for denitrification has been shown to affect process rates, sludge production, and denitrification yields (Henze, 1991). The next section investigated how feed protein type (fish meal and soybean meal) and inclusion level (P:E and % SBM) affected the types and masses of carbon produced through hydrolysis and fermentation. Section 2.4.1 describes the carbon net production dynamics of VFAs and ethanol produced in hydrolyzed and fermented sludge deriving from fish fed five fish meal based diets with increasing protein to energy ratios (P:E_15, 17, 19, 21 and 23 g/MJ). In section 2.4.2, the masses of the different carbon compounds from incubated sludge deriving from fish meal based and soybean meal based diets are measured and compared, and finally in section 2.4.3 the effect that each dietary treatment has on the denitrification capacity using the produced internal carbon sources are presented.

Experimental design

The SFS collected from two independent trials using feed with different protein type (fish meal and soybean meal) were used to evaluate and compare the composition and net production of readily available carbon sources under anaerobic conditions. In the first trial rainbow trout was fed five fish meal isoenergetic experimental diets with different P:E ratios (P:E_15, 17, 19, 21, 23) as presented in Table 3. In the second trial five iso-nitrogenous experimental diets with increasing concentrations (10, 20, 30, 40 and 50%) of solvent extracted, toasted, high-protein (48%) SBM at the expense of FM was evaluated (Table 5). A diet with FM as the sole protein source and an anticipated DP:DE ratio of 19 was included in the trial for comparison purposes.

Table 5. Ingredients and gross composition on the experimental diets

Diet	FM	SBM_{10}	SBM_{20}	SBM_{30}	SBM_{40}	SBM_{50}
Ingredients (%)						
Fish meal [1]	58.7	44.4	37.4	31.1	24.7	18.29
Soya Cake 48 Hi Pro Solvent Extr.	0.00	10.0	20.0	30.0	40.0	50.0
Wheat	23.6	36.7	35.0	30.0	24.0	18.5
Fish oil	18.6	11.7	12.5	14.3	16.1	18.0
Methionine	0	0	0	0.02	0.10	0.17
Vitamins & minerals [2]	0.30	0.30	0.30	0.30	0.30	0.30
Proximate composition (%) [3]						
Dry Matter	93.7	92.5	93.6	94.4	93.8	93.7
Protein	42.5	37.4	37.5	37.5	38.0	38.7
Lipid	25.9	17.8	17.3	19.9	20.5	20.9
Ash	9.9	8.0	7.6	7.1	6.7	6.3
Phosphorous	1.7	1.4	1.2	1.1	1.0	0.9
NFE (nitrogen free extracts) [4]	15.4	29.3	31.2	29.9	28.6	28.8
Gross energy (MJ/kg)	**22.1**	**20.3**	**20.4**	**21.1**	**21.3**	**21.6**

[1] SA 68 superprime Perú, South America (68% protein).
[2] Premix Dk 3, Biomar A/S Denmark.
[3] Analyzed as described in Dalsgaard and Pedersen (2011).
[4] NFE calculated as: dry matter – protein –lipid – ash.

The collected 4 day pooled SFS from both trials were independently hydrolyzed and fermented for 7 days in 1L anoxic/anaerobic reactors and maintained at 20 ± 2 °C with constant stirring at 200 rpm. Samples were obtained at day 0 for SFS characterization (TCOD, TKN and dry matter). Daily samples were taken for analysis of the RAC produced (VFAs and ethanol (Eth)). The characteristics of the SFS obtained from the fish meal and soybean trial are presented in Table 6 and 7 respectively. Data are presented as g masses produced/kg of fish produced.

Table 6. Characteristics (day 0) of settleable faecal solids (SFS) produced by rainbow trout fed diets with different fish meal (FM) protein:energy ratios (P:E). Data are based on 4 x 24 h sampling and pooling of SFS, values are expressed as masses produced/mass of fish produced (mean ± SD, n=3)[1].

Diet	P:E_15	P:E_17	P:E_19	P:E_21	PE:_23
Dry matter (kg/kg)	$0.15^{a} \pm 0.00$	$0.13^{a} \pm 0.01$	$0.12^{a} \pm 0.01$	$0.12^{a} \pm 0.01$	$0.13^{a} \pm 0.00$
TCOD (kg/kg)	$0.18^{a} \pm 0.01$	$0.13^{b} \pm 0.02$	$0.12^{b} \pm 0.02$	$0.11^{b} \pm 0.01$	$0.13^{b} \pm 0.00$
Protein (g/kg) [2]	$29.6^{a} \pm 1.8$	$30.6^{ab} \pm 1.8$	$33.0^{ab} \pm 3.5$	$34.0^{ab} \pm 1.5$	$35.4^{b} \pm 1.6$
Lipid (g/kg)	$24.0^{a} \pm 2.7$	$15.8^{a} \pm 3.6$	$16.1^{a} \pm 4.1$	$16.0^{a} \pm 4.1$	$18.5^{a} \pm 1.89$
NFE (g/kg)[3]	$82.6^{a} \pm 1.1$	$63.1^{b} \pm 4.8$	$54.0^{bc} \pm 5.7$	$48.1^{c} \pm 1.9$	$55.7^{bc} \pm 2.8$

[1] Values within rows not sharing a common superscript were significantly different (Tukey-Kramer, $P<0.05$).
[2] Protein was derived as total Kjeldahl nitrogen (TKN) multiplied by 6.25.
[3] Nitrogen free extract (NFE) was calculated as: NFE = dry matter – protein – lipid – ash.

Table 7. Characteristics (day 0) of SFS produced by rainbow trout fed different soybean meal (SBM) diets (plus FM control) and posteriorly used in the hydrolysis/fermentation batch study. Data are expressed as masses produced/mass of fish produced (mean ± SD, n=3), and are based on daily sampling and subsequent pooling for four consecutive days[1]

Diet	FM	SBM_{10}	SBM_{20}	SBM_{30}	SBM_{40}	SBM_{50}
Dry matter (kg/kg)	$0.13^{a} \pm 0.02$	$0.17^{ab} \pm 0.03$	$0.22^{bc} \pm 0.01$	$0.19^{bc} \pm 0.01$	$0.22^{bc} \pm 0.01$	$0.24^{c} \pm 0.03$
TCOD (kg/kg)	$0.20^{a} \pm 0.00$	$0.25^{b} \pm 0.01$	$0.31^{c} \pm 0.00$	$0.28^{d} \pm 0.00$	$0.32^{c} \pm 0.01$	$0.37^{e} \pm 0.01$
Protein (g/kg) [2]	$33.6^{a} \pm 2.9$	$37.8^{a} \pm 2.2$	$40.9^{a} \pm 3.8$	$36.1^{a} \pm 4.6$	$35.38^{a} \pm 3.1$	$38.3^{a} \pm 3.7$
Lipid (g/kg)	$17.6^{a} \pm 7.9$	$19.2^{a} \pm 1.5$	$23.1^{a} \pm 3.1$	$28.5^{ab} \pm 4.9$	$34.8^{b} \pm 7.4$	$40.0^{b} \pm 4.7$
NFE (g/kg) [3]	$56.9^{c} \pm 4.8$	$91.2^{ac} \pm 30.7$	$140.2^{b} \pm 8.8$	$115.6^{ab} \pm 7.3$	$131.6^{b} \pm 4.7$	$151.6^{b} \pm 20.9$

[1] Values within rows not sharing a common superscript were significantly different (Tukey-Kramer, $P<0.05$).
[2] Protein was derived from TKN by multiplying by 6.25.
[3] NFE was calculated as NFE = dry matter – protein – lipid – ash.

2.4.1 Effect of dietary P:E ratios on the production of individual readily available carbon compounds (RAC) (Paper II)

The results from the fish meal protein source trial showed that the distribution and quantities of VFAs and ethanol changed throughout the various days of fermentation as well as in relation to the dietary composition (Figure 6). In addition to ethanol (Eth), the following VFAs were identified in all groups: butyric (HBu), acetic (HAc), propionic (HPr), valeric (HVa), and formic acid (HFo). Regarding the carbon compound dynamics, a high production of acetic acid was observed during the first 24 h (43-62% of the total RAC identified) (Figure 6). Furthermore, the net production of acetic acid continued to increase in all groups (except for treatment group P:E_15) during the first 3-4 days of fermentation and then levelled out. According to the "Anaerobic Digestion Model No1" (ADM1) (Figure 7), acetic acid can be produced through several pathways (Batstone et al., 2002; Metcalf and Eddy, 2004; Henze et al., 2008), and this probably explains the high production in all treatment groups independently of the nutrient composition of the substrate (SFS).

Generally, the net production of the different VFAs changed after 2-3 days of fermentation when comparing the different treatment groups, indicating that the bacteria shifted to different pathways according to the substrate available. Hence, butyric acid was produced in particularly high amounts in the lowest P:E ratio treatment group, while a continuous high net production of acetic and valeric acid was observed in the highest P:E treatment groups (P:E_19, 21 and 23) (Figure 6). Propionic acid was produced in moderate amounts in all groups throughout the measuring period (Figure 6), while formic acid was the least produced RAC, being mainly produced during the first day of fermentation (Figure 6). Ethanol constituted 9-20% of the total RAC produced after day 1 in the different treatment groups, and there continued to be a net production until days 2-4, at which time it was replaced by a net consumption, except for treatment group P:E_15 (Figure 6).

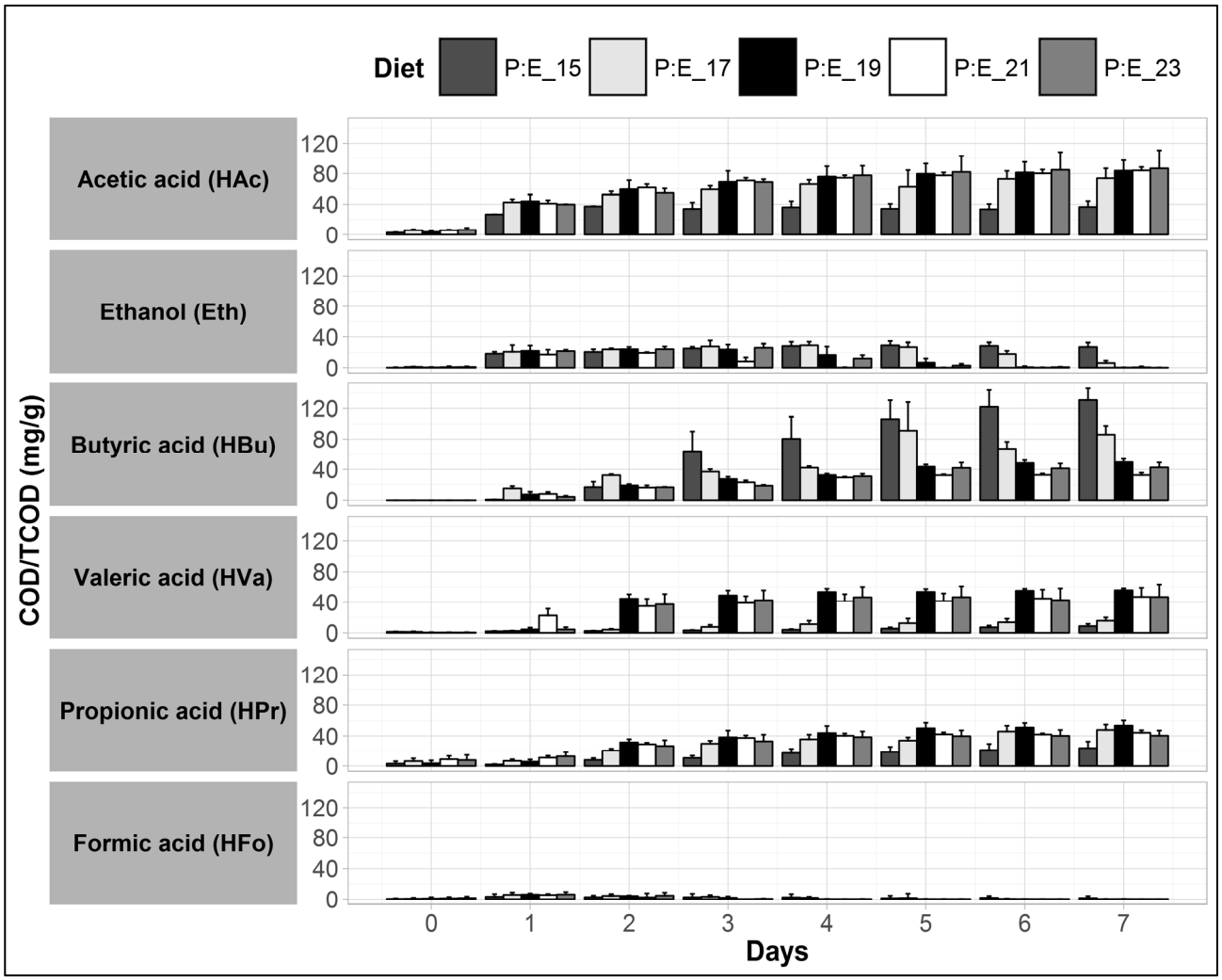


Fig. 6. Temporal pattern in the net production of **a)** acetic acid (HAc); **b)** ethanol (Eth); **c)** butyric acid (HBu); **d)** valeric acid (HVa); **e)** propionic acid (HPr) and **f)** formic acid (HFo) during 7 days of fermentation of the settleable faecal solids (SFS) deriving from different dietary treatment groups. Data are presented as COD values and normalized to TCOD measured in the samples (mean ± SD, n=3).

According to the ADM1 model (Figure 7), butyric and valeric acid can be produced from acidogenesis of sugars or amino acids. Acidogenesis of amino acids was thus probably the predominant bacterial pathway leading to the production of valeric acid, given that it was primarily recovered in organic waste from fish fed diets with the lowest content of carbohydrates and the highest content of proteins (diet P:E_19, 21 and 23). In contrast, acidogenesis of sugars was probably the main bacterial pathway leading to the production of butyric acid, as the production of this acid was highest in the treatment groups deriving from fish fed diets rich in nitrogen free extracts (NFE; i.e.carbohydrates; P:E_15 and 17).

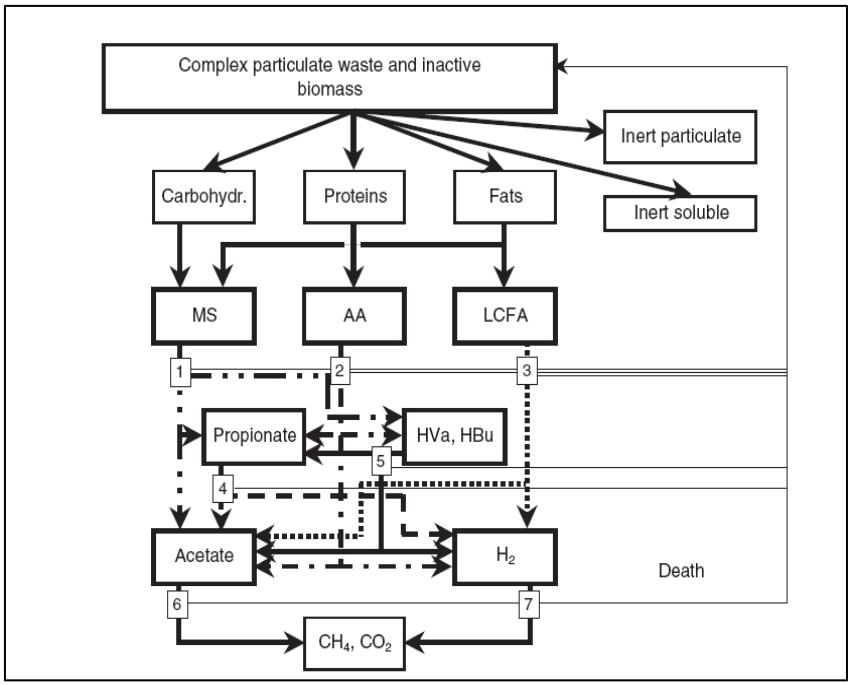


Fig. 7. The anaerobic model (ADM1) including biochemical processes: (1) acidogenesis from sugars (monosaccharide (MS)), (2) acidogenesis from amino acids, (3) acetogenesis from long-chain fatty acids LCFA, (4) acetogenesis from propionate, (5) acetogenesis from butyrate and valerate, (6) aceticlastic methanogenesis, and (7) hydrogenotrophic methanogenesis. Extracted from Batstone et al., (2002).

Accumulation of intermediate RAC compounds (butyric, valeric and propionic acids especially) observed in the current study sustained that an incomplete anaerobic process was taking place, corroborated by the daily dynamics of the individual VFAs and ethanol. Hence, acetic and formic acid are reduced end products in an anaerobic digestion process, and the consumption of formic acid, the stabilization of acetic acid net production, and the accumulation of intermediate organic acids are all major indicators of an incomplete anaerobic process. A complex food web is involved in a complete anaerobic digestion process, including a strict relationship between different bacteria. Since hydrogen producing acetogenic bacteria and methanogenic (hydrogen consuming) populations were most likely not well established in the reactors, an interspecies hydrogen transfer process was not fulfilled (Metcalf and Eddy, 2004; Henze et al., 2008). A low pH affects the free energy change (positive ΔG^0) and prevents the bacteria from further converting propionate and butyrate into acetate (McCarty and Smith, 1986). This probably explains the stagnant net production of acetic acid after the first three days and the simultaneous accumulation of intermediate organic acids including propionate and butyrate (Figure 8).

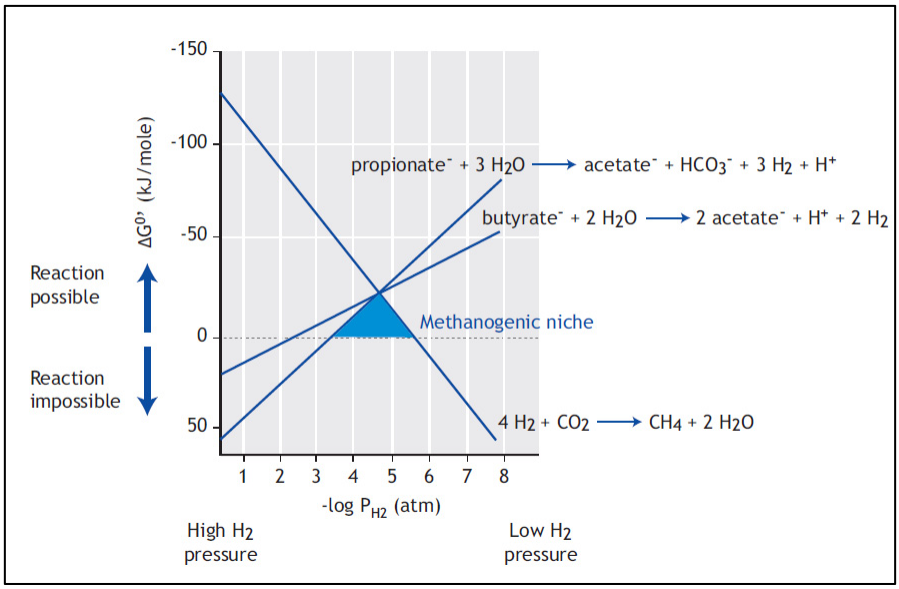


Fig. 8. Free energy change as a function of the H_2 partial pressure. A negative ΔG^0 indicates possible occurrence of the reaction. Extracted from Henze et al., (2008).

Identifying the different types of readily available carbon compounds that were produced from the different dietary treatments helped to understand that not only VFAs were produced from the fermentation process but also alcohols such as ethanol, which was most probably produced from the NFE fraction in the waste. Moreover, the fraction corresponding to alcohols probably explains the low degree of fermentation discussed in section 2.3.1 (and reported in paper I) as alcohols were not accounted for in the method applied. Similarly, knowing that different types of readily available carbon compounds influence the denitrification process in different manners (i.e., have different optimal C:N ratios), a more precise estimation of the capacity of each dietary treatment for pursuing denitrification can be performed.

2.4.2 Comparison of RACs produced in hydrolyzed and fermented organic waste deriving from fish fed fish meal or soybean meal based diets

The following section compares the different RAC produced from the hydrolysis/fermentation of the SFS produced from fishmeal and soybean dietary treatments. The net distribution of individual RAC compounds produced and the yields obtained (expressed as g RAC_COD/g TCOD) at the end of the 7 days fermentation process of organic waste deriving from fish fed FM and SBM based diets are presented in Figure 9a and 9b. The FM dietary treatments yields ranged between 0.21 and 0.24 g RAC_COD/g TCOD with no statistical difference between feed types. In the case of the SBM dietary treatments, values decreased from 0.18 g RAC_COD/g TCOD for SBM_10%, to 0.09 g RAC_COD/g TCOD for SBM_50%. The control P:E_19 diet used in the SBM experiment showed the same yield as SBM_10%, and no statistical difference in yields between feed types for the SBM dietary treatments was found.

The degree of fermentation, expressed as RAC_COD/sCOD, ranged between 72 - 91% for the fish meal dietary treatments, P:E_15 resulting in the lowest degree of fermentation and P:E_21 in the highest. While there was no clear tendency regarding effects of dietary protein levels in the fish meal dietary treatments, there was a decrease in the fermentation degree from 69 to 40% in the SBM dietary treatments as the level of SBM increased. The control diet (P:E_19) used in the SBM dietary treatment evaluation had the highest fermentation degree (86%) being significantly different compared to 40% in SBM 50%.

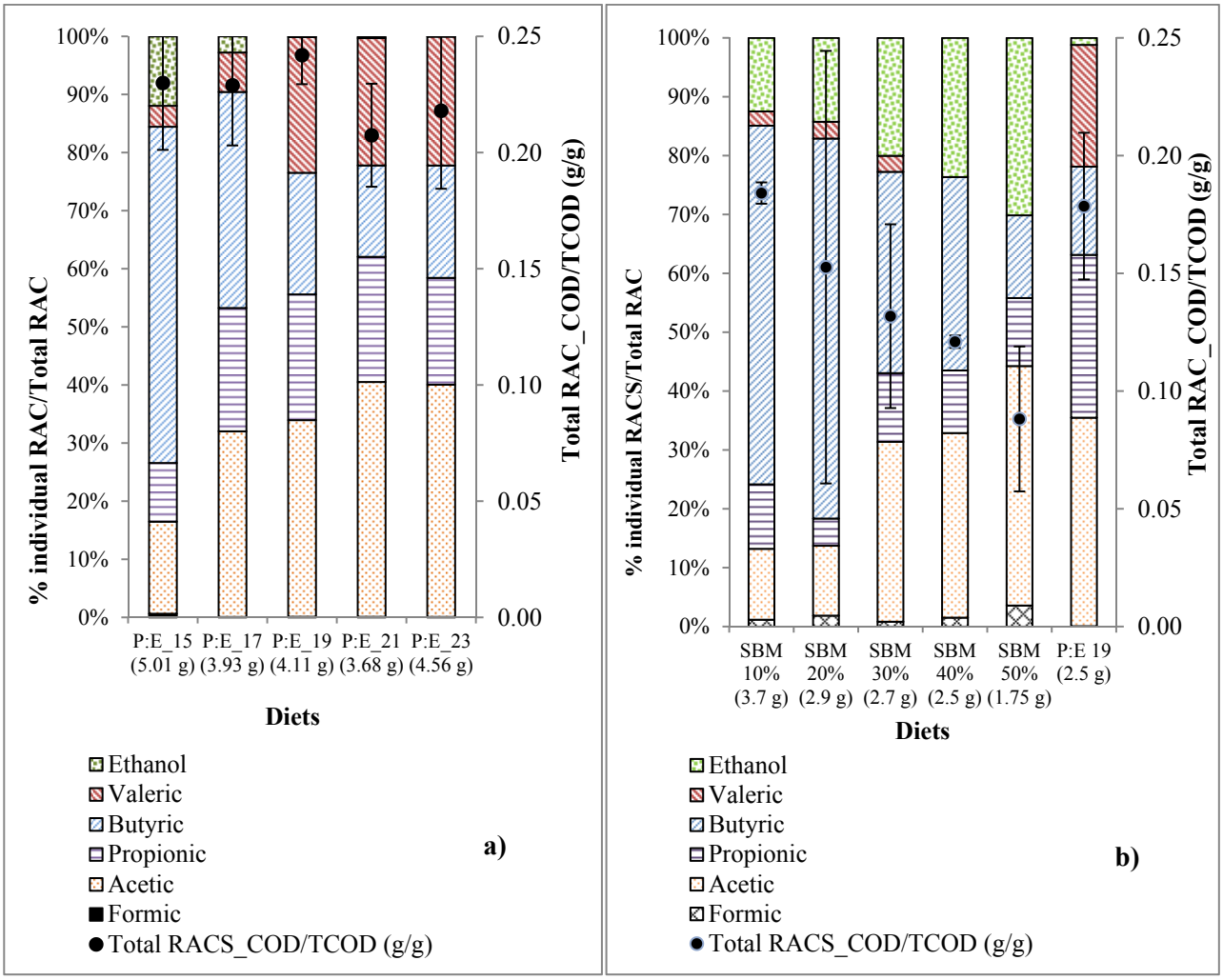


Fig. 9. Total quantities and composition of readily available carbon (RAC) compounds measured after 7 days of fermentation of the settleable faecal solids (SFS) deriving from different dietary treatment groups, fish meal (**a**) and SBM (**b**). The proportion of individual RACs is shown as % on the left axis (mean, n=3), while the total yields of RAC (g RAC/g TCOD) are shown on the right axis (mean ± SD, n=3). Obtained RAC (expressed as g COD/g wet sample) are displayed in brackets on the X axis. In the fish meal dietary treatments **(a)**, the yields of formic, acetic and butyric acid as well as ethanol in dietary treatment P:E_15 differed significantly from the other dietary treatments. Furthermore, the yields of valeric acid in dietary treatment P:E_19, 21 and 23 differed significantly from P:E_15 and 17. For the SBM dietary treatments (**b**), significant differences were found for the yields of acetic acid in dietary treatment P:E_19 compared to dietary treatment SBM_10 and 20%. Significant differences were found in the yields of propionic acids in P:E_19 compared to dietary treatments SBM_20, 40 and 50%. No significant differences were found between dietary treatments in the yields of formic acid. In contrast, significant differences were found in the yields of valeric acid and ethanol in dietary treatment P:E_19 compared to the SBM treatments.

Regarding the distribution of RAC produced, the fish meal dietary treatments P:E_21-23 resulted in the production of acetic acid as the main VFA, accounting for approximately 40% of the total RAC. In comparison, butyric acid was the main VFA produced in P:E_15 and 17 (60 and 37%, respectively). Valeric, propionic and formic acid were recovered in all groups in lesser amounts (4-23%, 10-22%, and ≤ 3%, respectively), while ethanol was recovered only in treatment groups P:E_15 -17 after 7 days (13 and 3% of total RAC, respectively) (Figure 9a). In the case of the SBM dietary treatments (Figure 9b), butyric acid was the main RAC found in the lower dietary treatments SBM_10% and 20% (61 and 50%, respectively). Dietary treatment SBM_30% and 40% produced mainly 33% of butyric acid, 31-32% of acetic acid and 21-

24% of ethanol, respectively. SBM_50% produced majorly acetic acid (42%) and ethanol (31%). The control (P:E_19), on the contrary, had a more diverse RACs production, majorly constituted of acetic acid (36%), propionic acid (28%), butyric acid (15%) and valeric acid (20%). Practically no ethanol was found in the control treatment after 7 days of fermentation (1%).

2.4.3 Production of carbon sources from the different dietary protein sources and estimated denitrification capacity

A mass estimation using equation 10 was carried out to express the results in absolute terms as masses of RAC produced per masses of fish produced for each protein source (Table 8 and Table 9). The estimations disclosed that fish meal based diets resulted in higher yields of RAC (22-23%) compared to soybean meal based diets (9-18%), which on the other hand resulted in higher and increasing shares of ethanol as the level of SBM in the diet increased. Even though SBM based diets resulted in more TCOD per unit fish produced (or in other words more organic waste), the lower fermentative capacity of the degraded COD reduced the potential for denitrification. Hence, there was a more favorable RAC:TN ratio in fermented sludge from the fish meal based diets (3.5-1.0 RAC/TN) compared to the SBM based diet (0.7-1.5 RAC/TN).

Table 8. Masses of readily available carbon (RAC) products obtained after 7 days of fermentation of the organic waste deriving from fish fed fish meal based diets with increasing P:E ratios.

			kg RAC[3]/ton fish produced								
Dietary treatment	**FCR[1]**	**TCOD/Fish produced[2] (ton/ton)**	**HVa**	**HBu**	**HPr**	**HAc**	**Eth**	**HFo**	**Total**	**RAC/TCOD (g/g)**	**RAC/TN (g/g)**
P:E_15	0.8	0.18	1.5	24.3	4.2	6.7	5.0	0.3	42.1	0.23	3.5
P:E_17	0.8	0.13	2.0	11.1	6.3	9.6	0.8	0.0	29.9	0.23	2.2
P:E_19	0.7	0.12	6.9	6.2	6.4	10.0	0.0	0.0	29.7	0.24	1.8
P:E_21	0.7	0.11	5.2	3.7	5.1	9.6	0.1	0.0	23.8	0.21	1.0
P:E_23	0.7	0.13	6.4	5.6	5.3	11.6	0.0	0.0	29.1	0.22	1.5

[1]Feed conversion ratios (feed consumed/biomass gain) obtained during the experiment (from Letelier-Gordo et al., 2015).
[2]Total COD (TCOD)/fish produced calculated using the yields of TCOD/feed consumed (from Letelier-Gordo et al., 2015) multiplied by the associated FCR obtained.
[3]HVa (valeric acid), HBu (butyric acid), HPr (propionic acid), HAc (acetic acid), Eth (Ethanol), HFo (formic acid).

Table 9. Masses of readily available carbon (RAC) products obtained after 7 days of fermentation of the organic waste deriving from fish fed diets with increasing inclusion levels of SBM.

			kg RAC[3]/ton fish produced								
Dietary treatment	**FCR[1]**	**TCOD/Fish produced[2] (ton/ton)**	**HVa**	**HBu**	**HPr**	**HAc**	**Eth**	**HFo**	**Total**	**RAC/TCOD (g/g)**	**RAC/TN (g/g)**
SBM_10%	1.1	0.23	1.0	26.3	4.7	5.2	5.4	0.5	43.1	0.18	1.5
SBM_20%	1.2	0.26	1.1	25.6	1.8	4.7	5.6	0.7	39.7	0.15	1.1
SBM_30%	1.1	0.25	0.9	11.4	3.9	10.2	6.7	0.3	33.5	0.13	1.0
SBM_40%	1.3	0.29	0.0	11.6	3.7	11.1	8.3	0.5	35.2	0.12	0.9
SBM_50%	1.4	0.34	0.0	4.2	3.4	12.1	9.0	1.1	29.8	0.09	0.7
P:E_19	0.8	0.13	4.8	3.5	8.2	6.4	0.0	0.3	23.3	0.18	1.0

[1]Feed conversion ratios (feed consumed/biomass gain) obtained during the experiment (Manuscript I).
[2]Total COD (TCOD)/fish produced calculated using the yields of TCOD/feed consumed (Manuscript I) multiplied by the associated FCR obtained.
[3]HVa (valeric acid), HBu (butyric acid), HPr (propionic acid), HAc (acetic acid), Eth (Ethanol), HFo (formic acid).

The denitrification potential of the different diets was estimated by applying the C:N ratios reported by Yatong (1996) and using the individual RAC yields obtained in the current studies (Table 8 and 9) as well as the TN produced from each dietary treatment (Figure 10a,b). The denitrification potential of the fish meal based treatments generally decreased as the P:E ratio increased, and as a result the capacity for removing all the N produced by the fish largely decreased from 176% to 52% (Figure 10a). Hence, even though the carbon yields (RAC/TCOD) between the fish meal dietary treatments were similar (Table 8), there were differences in the capacity for removing the N excreted by the fish, the C:N ratio of the lower P:E diets favoring the capacity while the C:N ratio of the higher protein content diets reduced the capacity. In the case of SBM dietary treatment, the denitrification potential decreased as the level of SBM inclusion increased (Figure 10b). SBM_10%, 20%, 30% and 40% had similar or higher capacity to remove the N produced (47-74% of the TN produced) than the control P:E_19 (48%), while SBM_50% had the most limited denitrification potential (35%). This trend reflects the low capacity of SBM dietary treatments to produce fermentable carbon compounds even though the amount of TCOD increased as SBM inclusion increased. In this sense, the findings align with those previously reported by Meriac et al. (2014) who found that comparatively more TCOD was produced by rainbow trout fed non-starch polysaccharide diets compared to starch based diets, while the biodegradability of the organic matter was significantly reduced.

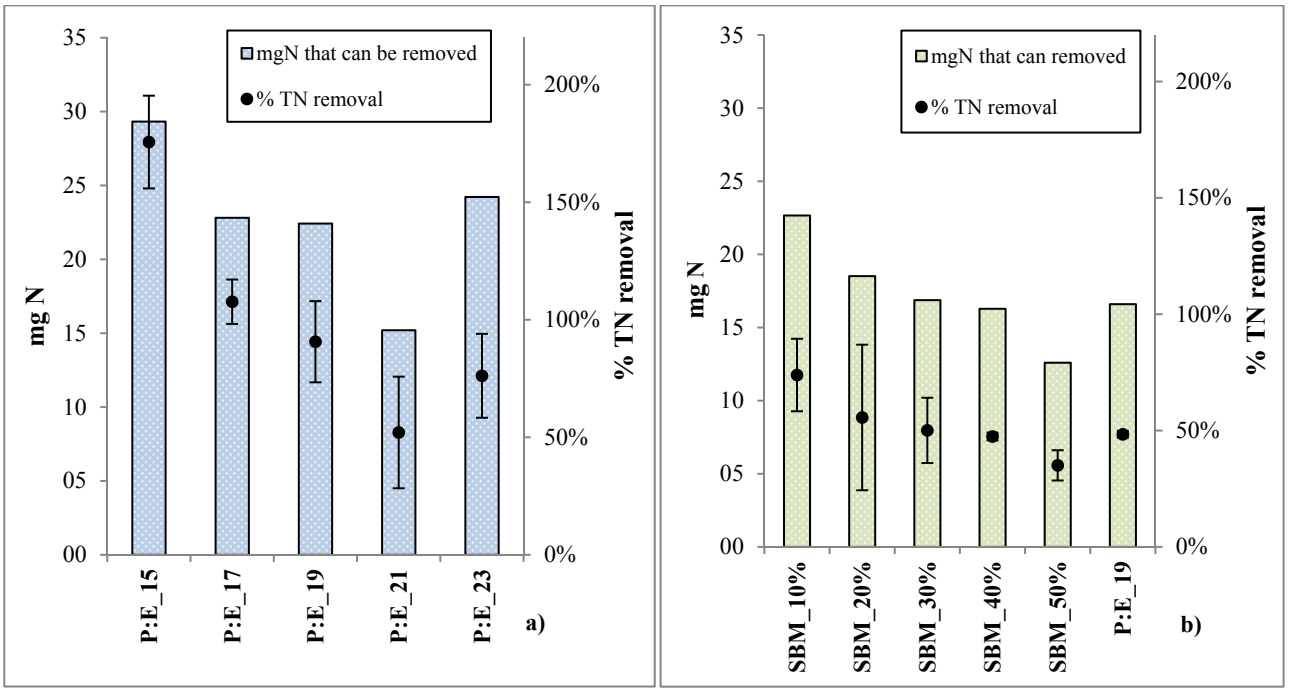


Fig. 10. Potential removal capacity of the total N excreted by fish fed either fish meal based diets (**a**) or soybean based diets and a control (P:E_19) diet (**b**). Primary Y axis shows mg N that potentially can be removed. Secondary Y axis show % of TN that can potentially be removed.

3. Part II: Optimization of the hydrolysis and fermentation processes

As previously mentioned, the degree of solubilization (sCOD/TCOD) obtained in the experiments was quite low, ranging between 0.24-0.30 g COD/g TCOD. In other words this means that only 24-30% of the total carbon available, measured as COD, became soluble and available for bacteria following hydrolysis of the solid waste under anaerobic conditions. In order to take full advantage of the capacity of fish waste for denitrification, the obtained yields must be increased. With this objective in mind, a series of experiments were conducted.

3.1 Hydrolysis under different environmental conditions

3.1.1 pH and inoculum

In Paper I, one of the hypotheses for explaining the shape of the solubilization degree curve was a possible feedback inhibition of the bacteria due to VFAs accumulating in the reactor after 3-4 days. It was hypothesized that the bacteria consortia, majorly coming from the intestine of the fish, exhibited a reduction in activity due to the low pH arising during the hydrolysis and fermentation processes (Hidalgo et al., 1998). When performing an incomplete anaerobic digestion process, methanogens will feed on VFAs in order to produce methane. In a complete anaerobic digestion process, hydrogen producing acetogenic bacteria and hydrogen consuming methanogenic populations will both be present and well established in the reactor, engaging in a symbiotic relationship (interspecies hydrogen transfer) which maintains pH between 7.5 – 8.5. As a result, feedback inhibition is avoided, and more than 80% degradation of the organic matter may be achieved in a well stablished reactor (Metcalf and Eddy, 2004; Henze et al., 2008; Mirsoyan et al., 2010; Mirsoyan and Gross, 2013). Considering the importance of pH on bacterial activity and the probable feedback inhibition due to the production and accumulation of VFAs, previous studies on the influence of pH on the process were reviewed. In a study by Chen et al. (2007), the hydrolysis process of wastewater was evaluated for 20 days under different pHs using activated sludge. The study showed that the production of VFAs was higher under alkaline conditions (pH 10) due to an increase in the rate of hydrolysis and an inhibition of methanogenesis activity. In a similar study, Wu et al. (2009) reported a higher solubilization of sCOD under alkaline conditions (pH 11) compared to lower pH values and uncontrolled pH. According to the authors, alkaline conditions resulted in the disassociation of acidic groups in the extracellular polymeric substances (EPS) of the sludge, thereby increasing protein and carbohydrate solubilization. On the other hand, Cokgor et al. (2008), using primary sludge from a wastewater treatment plant, reported a reduction in the hydrolytic capacity at pH values between 5.5 – 6.5, showing a delay in the acidification process and a lower VFA production.

According to the data obtained in the current study and the available information, two questions arouse: 1) how does pH affect the hydrolysis and fermentation process in fish waste; and 2) can the process be enhanced by inoculating a more robust consortia of bacteria from a previously established reactor. In order to resolve these questions, a pre-trial evaluating the hydrolysis and fermentation process during 96 h was performed.

Experimental design

Three consecutive trials were performed to evaluate the effect of pH and inoculum on the degree of solubilization (sCOD/TCOD) and the degree of fermentation (VFA/sCOD). The SFS were collected previous to the experiment from a laboratory RAS as described by Suhr et al. (2014) and immediately transferred to 1L anoxic/anaerobic reactors maintained at 21±2 °C with constant stirring at 200 rpm. In each trial three

reactors were evaluated in parallel, one batch reactor contained the collected SFS from the RAS and submitted to pH modification using a pH controller and chemical dosing pump. A second reactor served as control consisting of the SFS collected from the RAS with no pH adjustment. The third reactor contained a previous inoculum added at 20 % v/v and consisting of previous SFS collected from the RAS and adapted to the hydrolysis/fermentation process under alternating aerobic and anaerobic conditions (i.e., 12 h aeration, 12 h anaerobic) 48 h before the experiment. To avoid initial bacteria inhibition and giving time for the bacteria to adapt, pH was gradually changed to reach the required pH value during the first 24 hr. The trial period chosen (4 days) corresponded to the time where the degree of solubilization in the previous trials did not increase further. TCOD samples were taken at day 0 and daily samples of sCOD and VFA were taken every 6 hours during the first day of process and further with intervals of 1 day until the end of the experiment (day 4).

Effect of pH and inoculum on the degree of solubilization and the degree of fermentation

The results obtained after 96 h (Table 10) showed that the pH treatments (ph: 5, 7 and 9) had no effect on the degree of solubilization (sCOD/TCOD) when compared to the control (K) and inoculum (I) treatments. For pH 7 and 9, the yields obtained were similar to those previously reported, although for pH 5, an unidentified factor seem to have affected all the tested treatments including the control, since a yield of only 0.13 g sCOD/g TCOD was obtained. Regarding the degree of fermentation (VFA/sCOD), the only treatment that showed slightly higher values than the corresponding inoculum treatment and control was the pH 7 treatment group (0.58 g VFA/ g sCOD).

Table 10. Yields for degree of solubilization expressed as g sCOD/g TCOD and degree of fermentation expressed as g VFA/g sCOD obtained at day 4 from the different evaluated pH and inoculum conditions[1].

Trial	Treatment	Measured pH values[3]	TCOD (g/L)	sCOD/TCOD (g/g)	VFA/sCOD (g/g)
1st	**pH7**	7.3±0.06	10.6	0.18	0.58
1st	I[1]	6.2 ±0.1	11.6	0.15	0.51
1st	K[2]	6.0±0.0	11.5	0.14	0.51
2nd	**pH9**	9.2±0.1	6.9	0.18	0.65
2nd	I[1]	6.4±0.0	7.3	0.17	0.69
2nd	K[2]	6.3±0.0	7.7	0.17	0.73
3rd	**pH5**	4.7±0.0	10.1	0.13	0.32
3rd	I[1]	6.3±0.1	10.5	0.12	0.69
3rd	K[2]	6.3±0.0	11.0	0.12	0.73

[1]I: Treatment with inoculum.
[2]K: Control treatment.
[3]Measured pH values obtained during the 4 days trial (mean ± SD n=1)

Observing the dynamics of the VFA production, a constant pH of 7 reduced the process speed after two days, reaching the same maximum production of VFAs as the control and inoculum treatments (Figure 11a). In the case of pH 9, the highest production of VFAs was reached after 48 hr, similar to the production obtained in the control and the inoculum treatment (Figure 11b). In the following days (48-96 h), a consumption of VFAs was detected in treatment pH 9. In the case of pH 5, the production of VFAs was practically inhibited throughout the treatment period (Figure 11c). Interestingly, it seems that pH 5 affected only the fermentation

process and not the hydrolysis process when comparing the degree of solubilization between the control and the inoculum treatment (Table 10).

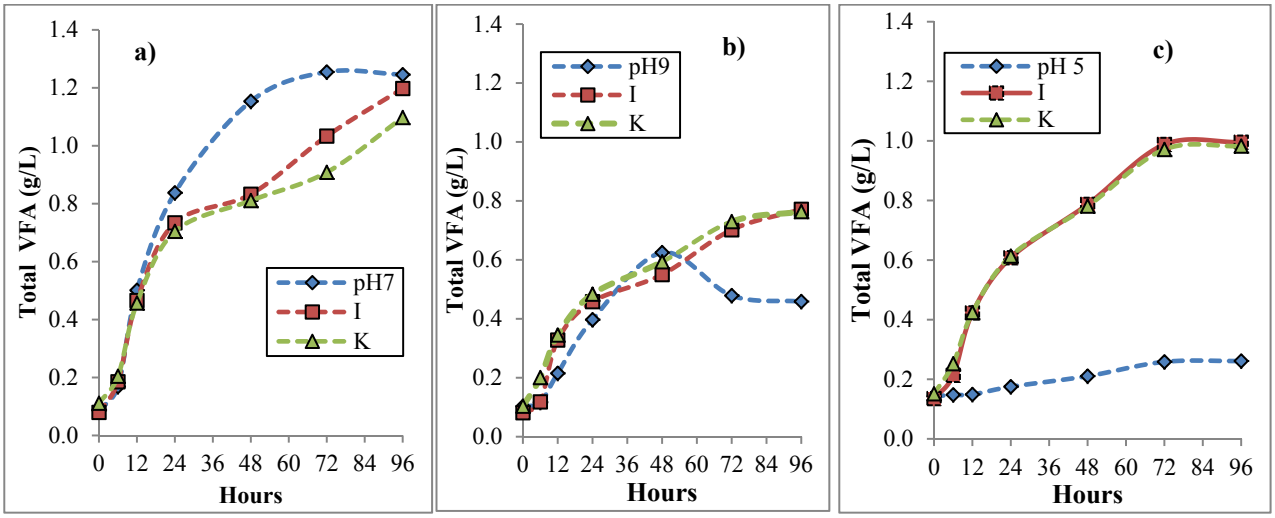


Fig. 11. Degree of fermentation found in reactor with pH modification to pH 7 (**a**, pH7), pH 9 (**b**, pH9) and pH 5 (**c**, pH5). I correspond to the inoculum treatments (no pH modification), and K are the controls (no inoculum no pH modification).

From the results obtained, it can be concluded that maintaining a pH of 7 can reduce the hydrolysis process time to only two days with similar yields as compared to the other pH treatments and the controls after 4 days. A low pH value (pH 5) affected the degree of fermentation but not the degree of solubilization (Table 10). Adding a previously developed inoculum from the same sludge did not improve the degree of solubilization or the degree of fermentation since similar trends as in the control were obtained.

The pre-trial thus showed that it was not possible to increase the degree of solubilization (sCOD/TCOD) by chemically changing pH. Thus other conditions hypothesized to affect the process were evaluated.

3.1.2 Temperature

Temperature is a well-known parameter affecting bacterial processes, with maximum bacterial growth rates normally achieved at higher temperatures (Henze et al., 2008). Yuan et al. (2011) evaluated the VFA production under different temperatures (4.0, 14.0 and 24.6 ^{0}C) and showed a strong effect of temperature on hydrolysis rate. A maximum production of sCOD was achieved in 5 days at 24.6 ^{0}C, whereas it took 7 and 9 days, respectively, to achieve the same yield at 14 and 4^{0}C. Cokgor et al. (2009) similarly evaluated the effect of temperature on the hydrolysis process using primary sludge, and reported a 5% increase in the degree of solubilization at 20^{0}C compared to 10^{0}C. With the aim of increasing the degree of solubilization obtained in the current study, the following experiment was carried out to evaluate the influence of temperature on the hydrolysis process.

Experimental design

Organic waste collected from sludge cones in a laboratory scale RAS as described by Suhr et al. (2014) was transferred to 1L anoxic/anaerobic batch reactors with constant stirring at 200 rpm. Three batch reactors (triplicate) were used to evaluate the effect temperature conditions, mesophilic temperature conditions

(40^0C) and a control at room temperatureor psychrophilic condition (20^0C), have on the degree of solubilization and the degree of fermentation. Samples for TCOD were obtained at day 0 and daily samples for sCOD and VFA were obtained at time 0, 4, 8, 24, 48, 72 and 96 h (Figure 12).

Effect of two temperature conditions (20 and 40 ^{o}C) on the degree of solubilization

Temperature was shown to have a strong influence on the rate of hydrolysis (Figure 12). In the 40^0C treatment, the maximum degree of solubilization was achieved in 48 h compared to 96 h in the control. The 40^0C treatment resulted in a 35% higher yield after 24 h compared to the control (Figure 12). Interestingly, after 48 h, the 40^0C treatment showed a consumption of sCOD.

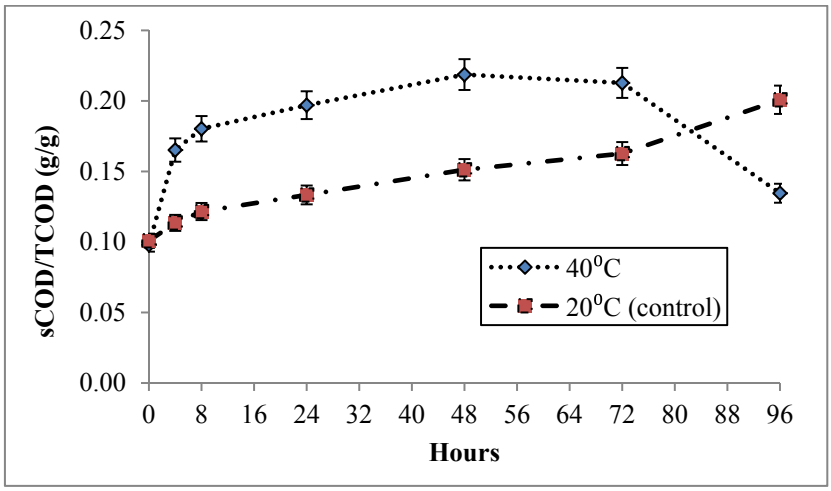


Fig. 12. Effect of temperature (mean ± SD, n=3) on the degree of solubilization (sCOD/TCOD) of fish organic waste incubated in anaerobic/anoxic batch reactors at 40^0C (mesophilic conditions), and at room temperature (20^0C) (psychrophilic conditions).

Temperature seems thus to have a direct effect on bacterial metabolism, as deduced from the graph, resulting in faster hydrolysis rates and consumption of the solubilized COD. This consumption of sCOD may be caused by the development of a methanogenic bacterial population, a situation that must be avoided when the objective is to produce VFAs for further use in the denitrification process. A reactor operated at 40^0C would reduce the time required to achieve maximum solubilization of organic matter, and would further reduce the volume required in the hydrolysis reactor to match the same hydraulic retention time.

The trial thus showed that at a temperature of 40 ^{0}C, the hydrolysis process rate doubled compared to the 20 ^{0}C as similar yields were obtained but with a 2 day difference. However, because there was a faster consumption of the end product (solubilized COD) at a temperature of 40 ^{0}C, it did not improve the maximal degree of solubilization (sCOD/TCOD) reaching approximately 0.2 at both temperatures.

3.1.3 Enzyme addition

Hydrolysis of particulate organic matter is considered to be a rate-limiting step in complete anaerobic digestion (Pavlostathis and Giraldo-Gomez, 1991; Vavlin et al., 2008). Adding enzymes to enhance the hydrolysis process has been evaluated as an alternative to increase sludge digestion in industrial and wastewater treatment. Parmar et al. (2001) reported a 45% reduction of organic matter (measured as Total suspended solids (TSS)) from sewage sludge using a combination of enzymes (protease and cellulase). In a similar study, Roman et al. (2006) assessed the impact of simultaneously adding cellulase and pronase E to

anaerobic sludge from methanogenic digesters, and achieved an 80% reduction in solids and a 93% removal of the particulate COD. In aquaculture sludge, Meriac (2014) evaluated the use of a commercial multi-enzyme complex (Viscozyme ® L) on faecal matter for improving the degradability of fibers found in the fish faecal matter. The author did not find any significant differences compared to the non-enzyme supplemented sample, and concluded that the remaining fiber fraction was resilient to degradation.

Because of these divergent findings, an experiment was carried out to investigate if the addition of enzymes could enhance the degree of solubilization (sCOD/TCOD) and VFA production compared to the previous experimental results.

Experimental design

The selection of enzyme was based on the proximate composition of the settable faecal solids described in paper II and reproduced in table 11. According to this, the major constituents of the faecal matter on a TCOD basis were proteins and NFE, and based on the results of Meriac mentioned above it was decided to focus the enzyme treatment on the protein fraction of the waste.

Table 11. Characteristics of settleable faecal solids (SFS) produced by rainbow trout fed diets with different protein:energy ratios (P:E) as described in Letelier-Gordo et al. (2015). Values are expressed as masses produced per masses of measured TCOD (mg/g; mean ± SD, n=3).

Diet	**P:E_15**	**P:E_17**	**P:E_19**	**P:E_21**	**PE:_23**
Dry matter	$840^{a} \pm 30$	$980^{a} \pm 30$	$1030^{b} \pm 90$	$1080^{b} \pm 60$	$990^{a} \pm 50$
Protein[2]	$162.3^{a} \pm 14.7$	$233.6^{b} \pm 18.0$	$272.9^{bc} \pm 26.9$	$296.8^{c} \pm 22.3$	$266.1^{bc} \pm 18.5$
Lipid	$131.0^{a} \pm 6.7$	$119.1^{a} \pm 15.6$	$130.9^{a} \pm 11.0$	$138.2^{a} \pm 21.8$	$139.3^{a} \pm 15.9$
NFE[3]	$317.1^{a} \pm 14.9$	$285.1^{a} \pm 12.0$	$218.0^{b} \pm 22.7$	$171.3^{c} \pm 12.9$	$194.5^{bc} \pm 13.0$
Ash	$230.6^{a} \pm 11.9$	$338.3^{b} \pm 16.8$	$407.2^{bc} \pm 51.6$	$472.6^{c} \pm 48.5$	$385.8^{bc} \pm 21.4$
TCOD (g/g)[4]	21.8 ± 2.7	17.2 ± 1.7	17.0 ± 3.7	17.7 ± 2.0	20.9 ± 1.4

[1] Values within rows not sharing a common superscript were significantly different (Tukey-Kramer, $P<0.05$).
[2] Protein was derived as total Kjeldahl nitrogen (TKN) multiplied by 6.25.
[3] Nitrogen free extract (NFE) was calculated as: NFE = total solids – protein – lipid – ash.
[4] Values correspond to g total chemical oxygen demand (TCOD)/g wet weight.

The enzyme applied (Neutrase 0.8 L from Novozymes; 1.6×10^{5} units/g protein) was a protease complex noted to be active in a pH range of 5.5 - 7.5 and a temperature between 30-50 ^{0}C. No modification of pH was necessary as the conditions registered during the hydrolysis fermentation process were within the required range of pH. However, to achieve a temperature within the enzyme working range, the 0.5 L anoxic/anaerobic reactors were located in a water bath shaker set at 35^{0}C. Different doses (% v/v) of Neutrase 0.8 L were applied to the reactors: 0% (control), 0.5%, 1.5% and 3.0%. Samples for TCOD were obtained at day 0 and samples for sCOD and VFA were obtained at day 0, 1, 3 and 5 after startup.

Effect of protease (Neutrase 0.8 L) on the degree of solubilization and VFA production

The obtained results showed no effect of enzyme addition on the degree of solubilization (Figure 13), and all treatments including the control reached similar values (0.26 - 0.31 g sCOD/g TCOD).

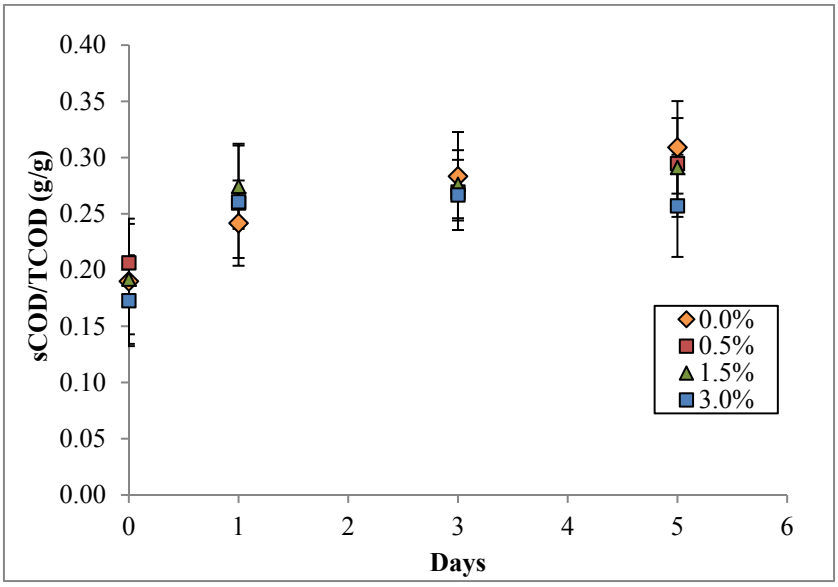
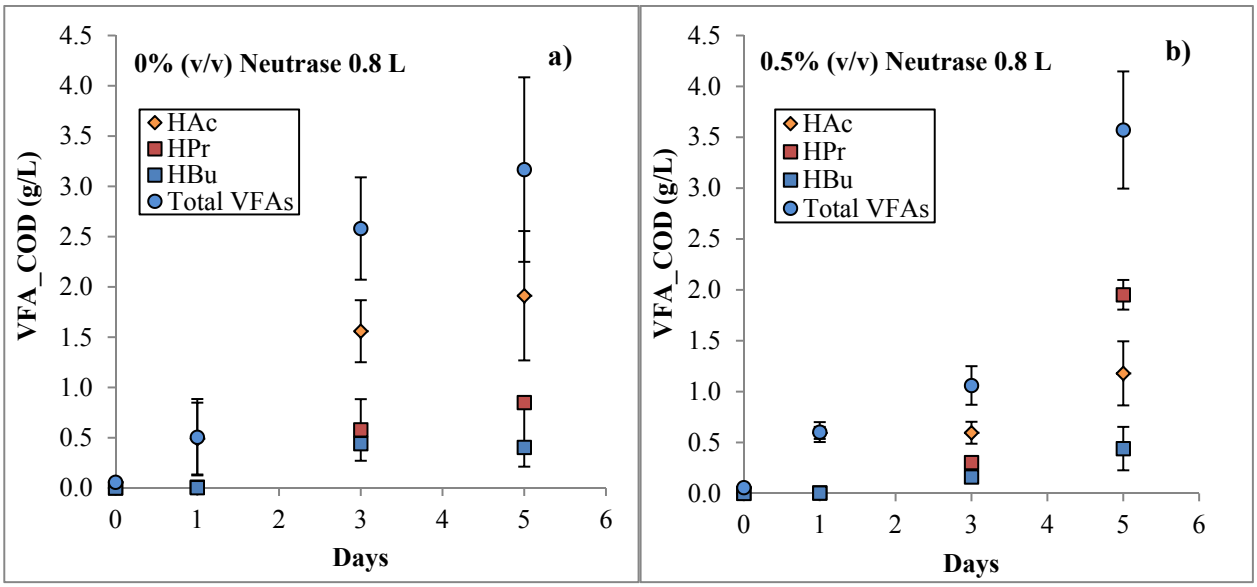


Fig. 13. Degree of solubilization (sCOD/TCOD) obtained from the addition of different doses (0.5, 1.5 and 3.0 % (v/v)) of Neutrase 0.8 L (mean ± SD, n=3) to the sample. The operational temperature was 35±3^{o}C for all treatments, and pH ranged between 7.5 - 6.2 in all reactors throughout the experimental period.

In contrast, the enzyme had negative impact on the fermentation process, and the net production of VFAs decreased as the inclusion of enzyme increased (Figure 14 a,b,c,d). Adding 0% (control) or 0.5% Neutrase 0.8 L to the reactors increased the production of VFAs compared to adding 1.5 and 3.0% of the enzyme. Acetic acid (HAc) and propionate (HPr) were the main VFAs found in all treatments.



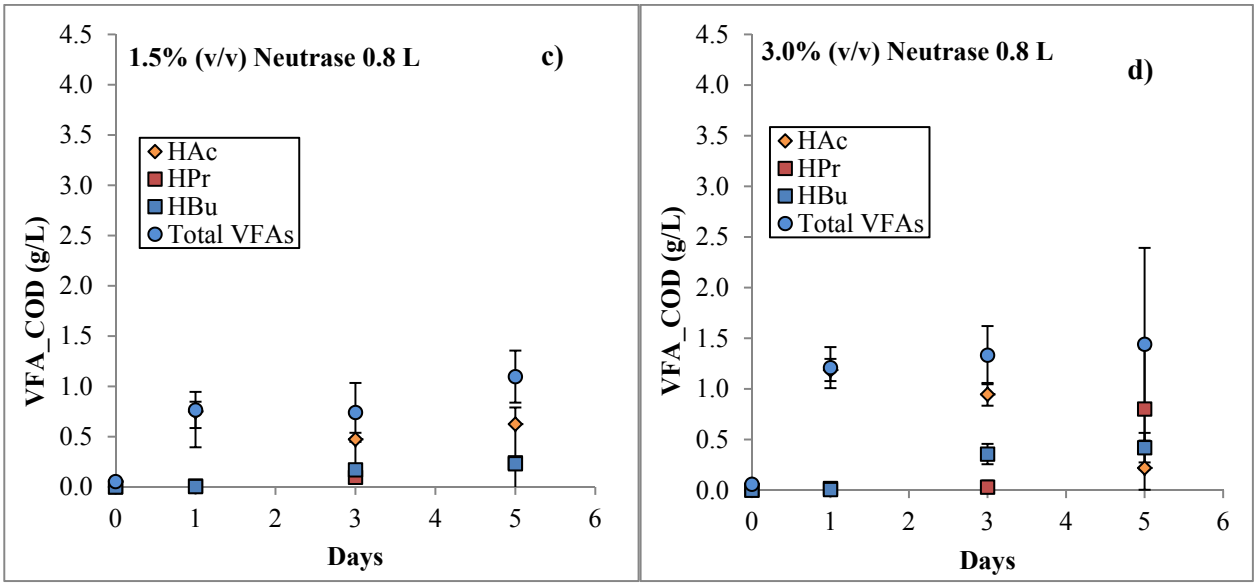


Fig. 14. Net VFA production dynamics (mean ± SD, n = 3) in batch reactors dosed with either 0 (**a**), 0.5 (**b**), 1.5 (**c**) or 3.0 (**d**) % (v/v) of Neutrase 0.8 L. The operational temperature was 35±3°C for all treatments, and pH ranged between 7.5 - 6.2. HAc (acetic acid), HBu (butyric acid), HPr (propionic acid),

In summary, the addition of protease did not improve the degree of solubilization (sCOD/TCOD). On the contrary, adding enzyme affected the fermentation process negatively. The reasons underlying these results are not clear. Presumably the chosen enzyme complex was not the adequate or the method for evaluating hydrolysis process was not either.

3.1.4 Reactor type: anaerobic sequencing batch reactor (ASBR)

The results obtained in the optimization part showed that the process speed could be increased through temperature or pH control, but that the degree of solubilization was unfortunately not similarly improved. The process curve generally reached a steady state at around 20-30% of hydrolysis, sustaining the hypothesis that some kind of inhibition might occur. All the experiments so far had been carried out in a batch type reactor, and it was speculated whether employing an anaerobic sequencing batch reactor (ASBR) could provide for an improvement in the hydrolysis-fermentation process. An ASBR configuration comprises 4 major phases: (1) fill, (2) react, (3) settle and (4) draw (Metcalf and Eddy, 2004). The reactor has mainly been applied in studies with slow growing organisms. It has proven to be a powerful experimental set-up as it efficiently retains biomass, creates a homogenous distribution of substrates and products, and has a reliable operation and stable conditions in substrate-limiting conditions (Dague et al., 1992).

The hypothesis that an ASBR could perform better than the batch type reactor was based on the fact that by replacing the supernatant of the reactor, the components potentially creating the feedback inhibition would be diluted, thus allowing the bacteria to recover and continue the hydrolysis process. Additionally, bacterial wash-out could be avoided. For example, if an inhibitory concentration of H^+ ions was reached after a certain time, an exchange of water (draw phase) and the addition of a new volume of water to the system (filling) would aid the bacteria in increasing the degree of solubilization. In the previous experiments, the sCOD production curve increased semi-exponentially during the first 2 days, reaching a maximum of 20% sCOD at day 4, followed by a sCOD net consumption starting at day 8. Therefore, the following experiment evaluated different operational cycles in ASBR (2, 4 and 8 days) using anoxic water from a RAS.

Experimental design

Sludge collected from sludge cones in a laboratory scale RAS as described by Suhr et al. (2014) was transferred to 1L anaerobic sequencing batch reactors (ASBR). Three different operational cycles were evaluated (2, 4 and 8 days) in triplicate during 8 days. Thus, for a 2 day operational cycle 4 runs were evaluated, for a 4 day cycle 2 runs were evaluated and for 8 day cycle only one run was evaluated. Each operational cycle consisted of 4 phases 1) filling 2) reaction 3) settle and 4) draw. The filling and draw phases was done using a peristaltic pump taking 5 minutes to fill or draw, respectively, 80% of the reactor volume in each cycle. Milli-Q water previously bubbled with N_2 gas to remove dissolved oxygen was used to replace the supernatant removed in each draw phase, maintaining the reactor volume at 1L. The settling phase was conducted 1 hour before sampling by turning off the magnetic stirrer. Initial samples of TCOD were taken at day 0 while samples of sCOD and VFA were taken according to the operational cycles of each reactor. In order to compare the performance between the reactors at different days, the data was analyzed by the mass of sCOD and VFA discharged from each corresponding operational cycle.

Effect of ASBR on the degree of solubilization and the production of VFA

The results showed that by operating the reactor at an operational cycle of 2 days, a slight increase in the degree of solubilization was obtained although the yield did not differ significantly from treatments operated at 4 and 8 days cycle. Moreover, the yields were similar to those obtained in the previous experiments (Figure 15a). The masses of VFA recovered from the reactors operated with a 2 days cycle were 19% higher than that of the reactors operated with a 4 days cycles (not significant different) while a significant 70% higher VFA mass production was obtained in operational cycle of 2 days as compared to the 8 days cycle (Figure 15b).

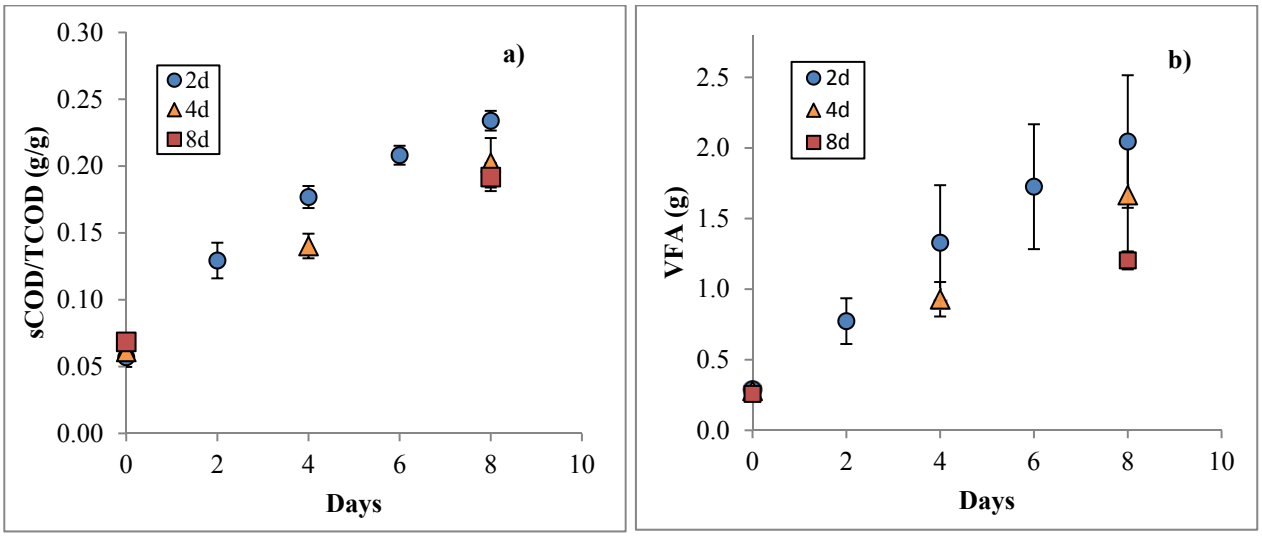


Fig. 15. Degree of solubilization (**a**) and cumulative masses of VFA produced (**b**) in RAS SFS incubated in anaerobic sequence reactors working at different operational cycles (2, 4 and 8 days) (mean ± SD, n=3).

As a brief conclusion the application of an ASBR and by this diluting potential inhibitors in the reactor did not show to improve the overall dissolution process. Indicating that probably the bacteria was inhibited without capacity for recovering between operational cycles or that eventually a different group of bacteria which was not present in the reactors is required to proceed with the dissolution process.

Summary of Part II

In summary, the different optimization studies showed that:

- None of the different methods applied for optimizing the degree of solubilization were able to solubilize more than 20-30% of the total COD.

- An increase in temperature to 40^{o}C (mesophilic conditions) or a constant pH of 7 halved the time required for achieving the same yields of VFA , therefore also halving the required volume of the reactor given the same HRT.

- Further research should focus on understanding the most optimal environmental and operational conditions for the bacteria in order to increase the degradation yield while at the same time avoiding/reducing methanogenesis. Carbohydrate, lipid and protein degradation properties and possible inhibition effects on the bacteria should be studied and eventually optimized in order to obtain the maximum potential from the particulate/fish waste as an internal carbon source for denitrification.

4. Part III: Applicability of internal carbon sources for denitrification on a Danish brood stock farm: a mass balance approach (Manuscript II)

The following part of the dissertation evaluated the application of a side stream fermenter (SSF) to enhance the production of readily available carbon for denitrification using the discharged organic matter from a low intensity, partly recirculated Danish brood stock farm (Manuscript II).

4.1 Study site

The study was carried out at a Danish rainbow trout brood stock farm located in the northern part of Denmark, comprising a hatchery and 10 earthen ponds/raceways (Figure 16). The farm has an internal flow of 8208 m^3/d from which 6048 m^3/d are recirculated back to the fish tanks after being treated using sludge cones and a fixed bed biofilter. The non- recirculated flow (2160 m^3/d) is lead through a larger end-of-pipe biofilter (fixed bed, 81 m^3) with Bio-Blok® (200 m^2/m^3) before entering a very small constructed wetland for final polishing before discharge into a stream.

Fig. 16. Hatchery and nursery (building at the back) and earthen ponds with rainbow trout brood stock

4.2 Trial Setup

To evaluate the applicability of a side stream fermenter (SSF) under commercial scale conditions, water from the backwash of a 60 µm drum filter (#1 in Figure 17) was used to supply organic waste to the SSF. The SSF consisted of 2 parallel cylindrical concrete tanks each of a volume of 11.9 m^3 (#2 in Figure 17). The SSF were connected in parallel and worked alternately in order to deliver a constant supply of organic waste through a distribution pump (#3 in Figure 17) into the denitrification reactor (#4 in Figure 17). The SSF was filled with organic waste derived from the cleaning water from the hatchery tanks (Letter A in Figure 17), the sludge cones (Letter B in Figure 17) and the backwash from the two biofilters (Letter C and D in Figure 17). Water from the 81 m^3 fixed biofilter overflow (Letter D in Figure 17), was pumped into a moving bed denitrification reactor (#3 in Figure 17) thereby providing a constant supply of water containing NO_3^-. The denitrification reactor had a volume of 20.3 m^3 and was filled 50% with RK BioElements (750 m^2/m^3, 10m^3).

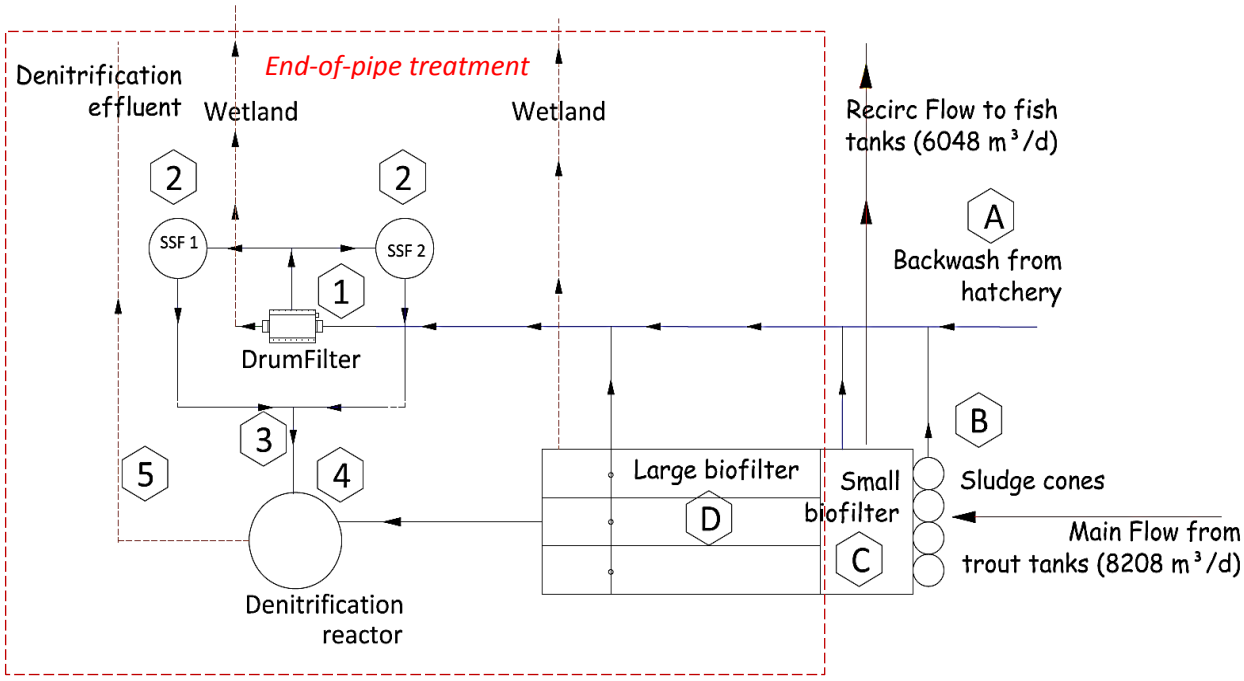


Fig. 17. Diagram of the end-of-pipe treatment (red dashed lines: (1) drum filter (2) side stream fermenters (SSF); 11.9 m^3 each (3) distribution pump (4) denitrification reactor 20.3 m^3 moving bed (5) effluent from the denitrification reactor into the wetland. The organic waste for the SSF were obtained from: (A) backwash and flushing of hatchery, (B) sludge cones, (C) backwash of small biofilter (44 m^3 fixed bed), and (D) large biofilter (81 m^3 fixed bed).

4.2.1 Systems mass balance method

The performance of the SSF and denitrification reactor was evaluated by applying a mass balance approach (equation 11 and 12). The SSF was defined as a control volume with IN masses corresponding to water discharged from the backwash of the drum filter, and OUT masses corresponding to water discharged into the denitrification reactor (Figure 18). Similarly, the denitrification reactor was defined as the control volume where IN corresponds to the masses entering the reactor, namely the water pumped from the large biofilter and the water discharged from the SSF, and OUT is the masses discharged from the denitrification reactor into the constructed wetland (Figure 19).

$$V * \frac{dC_{SSF}}{dt} = Q_o * C_{SSF,o} - Q * C_{SFF,effluent} + r_{SSF} * V \qquad \textbf{eq. 11}$$

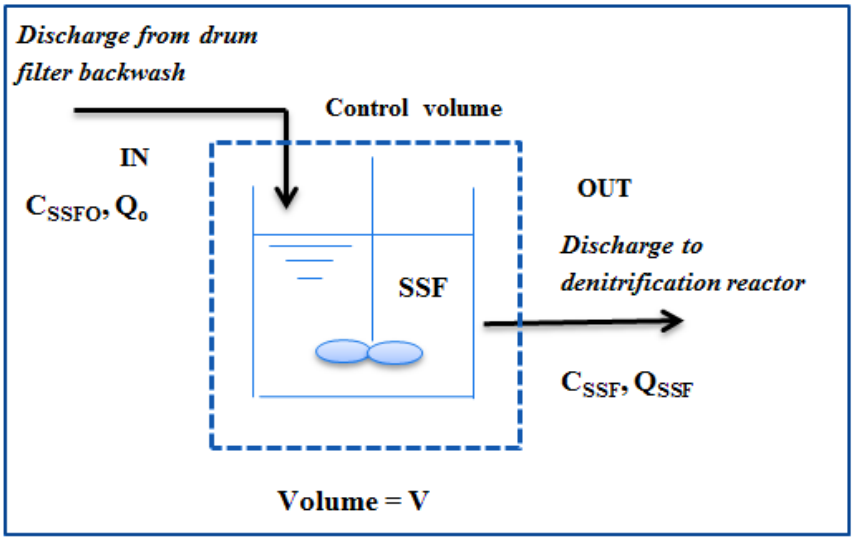


Fig 18. Mass balance of the side stream fermenter (SSF).

Where:
dC_{SSF}/dt = rate of change of reactant concentration within the control volume (g/m^3*d)
V = reactor volume (control volume) (m^3)
Q_0, Q_{SSF} = volumetric flow rates (m^3/d)
C_{SSFo}, C_{SSF} = concentration of SSF in the influent and effluent (g/m^3)
r_{SSF} = volumetric reaction rate (generation or consumption rate) (-1/d)*(g/m^3)

$$V * \frac{dC_{Deni}}{dt} = (Q_{SSF} * C_{SSF} + Q_B * C_{Bio}) - Q_{Deni} * C_{Deni,effluent} + r_{Deni} * V \qquad \textbf{eq. 12}$$

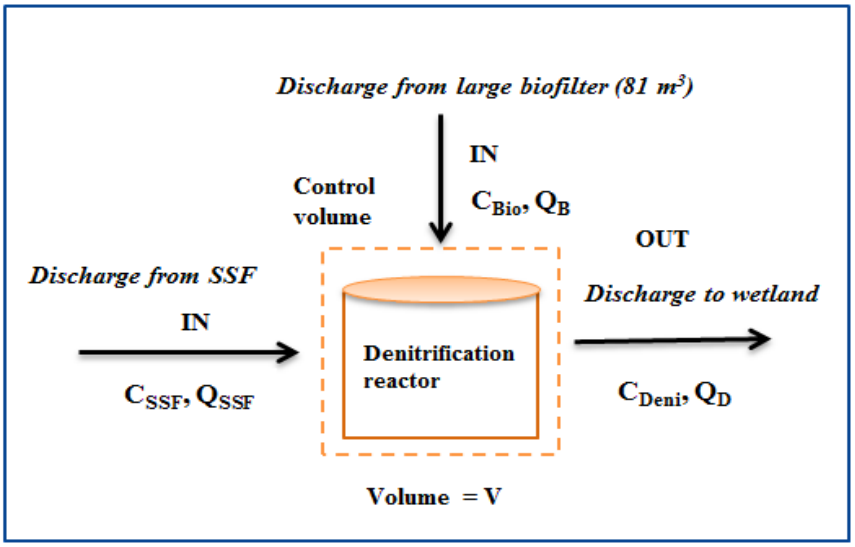


Fig. 19. Mass balance of denitrification reactor

Where:
dC_{deni}/dt = rate of change of reactant concentration within the control volume (g/m^3*d).
V = reactor volume (control volume).
Q_B, Q_{SSF}, Q_{Deni} = volumetric flow rates (large biofilter, side stream fermenter and denitrification reactor) (m^3/d).
C_{SSF}, C_B, C_{Deni} = concentration of side stream fermenter and biofilter in the influent and the denitrification reactor at effluent (g/m^3).
r_{Deni} = volumetric reaction rate (generation or consumption rate) (-1/d)*(g/m^3).

4.2.2 Characterization of the organic waste flows

A flow characterization was developed in order to estimate the amount and quality of carbon that was recovered from the weekly cleaning routines at the farm, and which subsequently feed the SSF (Table 12).

Table 12. Flow values (m^3/d) from the different sources of organic matter feeding into the SSFs on different days of the week.

Flows	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday	Total volume
	(m^3/d)	(m^3/d)	(m^3/d)	(m^3/d)	(m^3/d)	(m^3/d)	(m^3/d)	**(m^3/wk)**
Hatchery	0.05	0.05	0.05	0.05	0.05	0.05	0.05	**0.35**
Sludge cones	0.09	0.09	0.09	0.09	0.09	0.09	0.09	**0.63**
Small Biofilter	0.43	0.43						**0.86**
Large Biofilter			1.54					**1.54**
Sub total	**0.57**	**0.57**	**1.68**	**0.14**	**0.14**	**0.14**	**0.14**	**3.38 m^3/week**

From the flow characterization, an input of 3.36 m^3/week of sludge entering the SSF was recorded. For evaluation purposes and to ensure a constant input of flows and type of sludge, the flow discharged from the SSF to the denitrification reactor was set to 0.48 m^3/d, corresponding to a HRT in the SSF of 7 days. According to the amount of organic waste that was recovered, the denitrification reactor was successively set to operate under three different flows: 6, 18 and 54 m^3/d.

4.2.3 The trials and sampling procedure

The trial lasted 42 days where each selected flow in the denitrification reactor (6, 18 and 54 m^3/d) was evaluated during 14 days. Samples from each organic waste type (hatchery, sludge cones and biofilters) were obtained weekly according to the cleaning protocol of the devices (Table 12) and transferred to laboratory. The samples of each of the organic waste types characterized for their composition (N, P and organic matter) and degradability under anaerobic laboratory conditions to determine the quality of the sludge. Simultaneously, 24 h pooled samples with a sampling frequency of an hour were taken every two days in: 1) the water pumped from the 81 m^3 fixed biofilter into the denitrification reactor (Letter D in Figure 17) 2) the water pumped from the SSF (#3 in Figure 17) into the denitrification reactor (#4 in Figure 17) and 3) the water discharged from the denitrification reactor (#5 in Figure 17). All samples were taken with an automatic portable sampler and refrigerated at 4^{0}C before transferring them for laboratory analysis. A characterization of the quality of each organic waste type (hatchery, sludge cones and biofilters) was evaluated during 7 days to simulate the HRT set in the SSF in laboratory conditions. For this purpose 1 L batch anaerobic/anoxic batch reactors were operated at a temperature of 20.3 ±2^{0}C with constant stirring (200 rpm). Daily samples were obtained for analyses of TAN, PO_4^{3-}-P, NO_3^--N, NO_2^--N, VFAs, and sCOD. At the same time, pH and temperature were monitored daily. TCOD, TP, and TKN were measured in the reactors at the start of the anoxic/anaerobic degradation period (day 0).

4.3 Quality of the sludge obtained

The organic waste characterization for C, N and P forms obtained from the backwash of the drum filter from the different sources (hatchery, small biofilter, large biofilter and sludge cones) are presented in Table 13. According to the results obtained from the 7 days degradability laboratory trials, the degree of solubilization ranged between 1.4-10.6% (14.0 – 106 mg sCOD/g TCOD) (Figure 20a) while the degree of

fermentation ranged between 12-22% (0.12 – 0.22 mg VFA_COD/mg sCOD) (Figure 20b). The hatchery and the small biofilter showed increased values for these two parameters reaching VFA concentrations of 18.7 ± 5.9 mg VFA_COD at day 2 and 17.4±2.1 mg VFA_COD at day 3, respectively. The lowest degradability was found for the large biofilter backwash, only reaching a degree of fermentation of 0.12 ± 0.01 at day 1 with a production of 5.16 mg VFA_COD/mg sCOD at that same period of time.

Table 13. C, N and P composition of the different organic waste sources used in the SSF (mean ± SD, n=3).

Device	Carbon			Nitrogen				Phosphorous	
	TCOD g/L	sCOD mg/L	VFA mg/L	TN mg/L	NO_3^--N mg/l	NO_2^--N mg/L	NH_4^+-N mg/L	TP mg/L	PO_4^3-P mg/L
Hatchery	0.8±0.2	48.3±27	2.3±0.5	48.1±13.2	4.7±0.4	0.6±0.0	1.6±0.6	19.4±4.4	0.5±0.0
Sludge cones	1.6±0.4	24.5±6.1	2.2±1.3	80.4±17.4	5.0±0.0	0.1±0.1	1.3±0.8	61.4±11.1	0.2±0.1
Small Biofilter	3.3±0.7	34.6±19.8	1.6±0.8	170.8±33.6	4.7±0.5	0.6±0.4	1.0±0.3	96.4±21.5	2.7±2.9
Large Biofilter	2.3±0.4	15.7±2.5	1.0±0.4	128.3±22.7	5.1±0.4	0.3±0.0	0.6±0.4	78.5±12.6	0.3±0.1

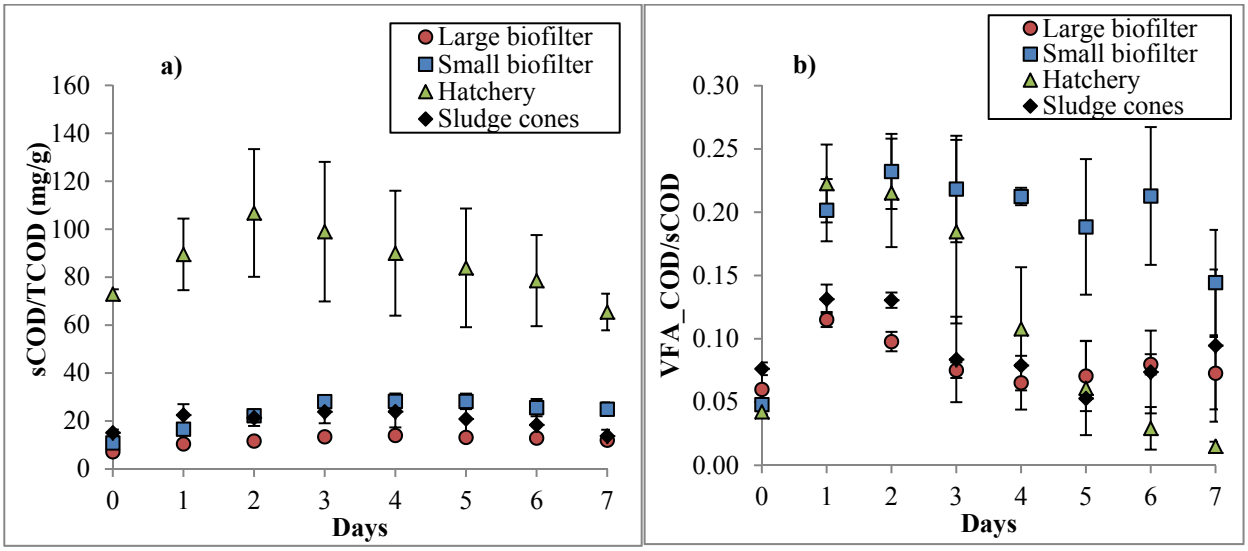


Fig. 20: Degree of dissolution (sCOD/TCOD) found in the different organic waste types during 7 days of anoxic/anaerobic degradation performed at laboratory conditions (**a**) (mean ± SD, n=3)**. b)** Degree of fermentation (VFA_sCOD/sCOD) from different organic waste types during 7 days of anoxic/anaerobic degradation performed at laboratory conditions (mean ± SD, n=3). The temperatures in all trials were 20.3±2 ^{0}C.

According to the results obtained under laboratory conditions, the degradability of the organic waste is similar to described by Ucizik and Henze (2008) for degradability of organic matter obtained from an activated sludge system in a wastewater treatment plant, indicating the highly degraded state of the organic waste presumably composed of bacterial mass. The low organic waste quality relates mainly to the farm configuration, as the organic waste have been submitted to saturated oxygen water conditions, long passage and long retention times in the farm water circuit before its collection. Because of this the easily degradable fraction of the organic matter has already been "lost" (dissolved) or consumed by bacteria before entering the SSF. Additionally, the collection method (drum filter) collected only the particulate fraction above 60 μm while the smaller particles and the soluble fraction was lost passing through the drum filter mesh. Comparing the degree of solubilization obtained in this trial with other studies from aquaculture organic waste (section 2.3.1; Figure 3a) from waste collected in settling cones (230-300 mg sCOD/g TCOD), the values obtained

here were some 10 times lower (~23 mg sCOD/g TCOD). Conroy and Couturier (2009) reported 400 mg SCOD/ g TVS from waste collected in swirl separators and Suhr et al. (2012) reported 200-300 mg sCOD/g TCOD for different organic waste (sludge cones and drum filter backwash). This difference stresses the importance of how the waste is collected and the time it has spent in the RAS treatment circuit for realizing its full potential as internal carbon source.

4.4 Performance of the SSF

The nutrients and organic matter forms expressed as concentrations in the discharge of the SSF illustrate the process stability during the experimental period (42 days) (Table 14). Total COD values averaged 2.9 ± 1.4 g/L, showing variability due to the properties of the sludge. The dissolved organic matter concentrations were more stable with sCDO values of 68.6 ± 19.9 mg/L and VFA averaged 23.5 ± 4.2 mg/L. NO_3^--N and NO_2^--N discharged from the SSF were constantly below the detection limits whereas NH_4^+ and PO_4^{3-}-P were produced at quite constant concentrations of 13.1±4.5 mgNH_4^+-N/L and 3.4±0.5 mg PO_4^{3-}-P/L. From the measured VFAs, acetate was by far the main compound averaging 22.9±3.9 mg/L corresponding to 97.4% of total VFA measured. The degree of solubilization was 2.3% (0.23 mg sCOD/mg TCOD) while the obtained degree of fermentation was 34% (0.34 mg VFA/ mg sCOD). The pH values measured inside the reactor during the trial were 7.0 ± 0.1 with a temperature of 12.9 ± 0.9ºC and dissolved oxygen concentrations <0.2 mg/L.

Focusing on the degree of fermentation (VFA/sCOD) it should be noted, that the VFA yield obtained in the SSF were higher (34%) as compared to the values obtained in the laboratory (10-18%). Most probably, methanogenic or sulfate reducing bacterial populations established in the SSF creating suitable conditions for acetogenic bacteria to degrade short-chained fatty acids (i.e. propionate and butyrate) into acetate. This was also reflected by the fact that majorly acetate and in some extend formate (2.6% of total VFAs) was measured in the SSF, while in the laboratory trials butyrate, valerate, propionate, acetate and formate accumulated in the batch reactors. Methanogenic and sulphate reducing bacteria could have utilized molecular hydrogen, creating a syntrophic association with the acetogenic bacteria (interspecies hydrogen transfer) allowing acetogenic bacteria to degrade short-chained fatty acids to acetate under exergonic conditions (energetically favored) (Henze et al., 2008; Muyzer and Stams, 2008). Additionally, low concentrations of sulfate were found in the SSF as compared to the surrounding water (1.3±0.3 mg SO_4^-S/L vs 5.3±0.1 mg SO_4^-S/L). Also more stable pH values (7.0 ± 0.1) were found in the SSF as compared to the laboratory trails (values averaged 7.4 ± 0.3 at day 0 and decreased to 6.6 ±0.1 until day 7) reinforcing the suggestion of a well-developed anaerobic digestion process being established in the SSF.

4.5 Denitrification system performance under different flow conditions

The performance of the denitrification reactor depended on the operational flows as seen on the NOx concentrations found in the effluent of the reactor (Table 14). The NO_3^--N concentration entering the reactor was relatively constant (5.4±0.4 mg-N/L), while the effluent concentration varied according to the operational flows of 6, 18 and 54 m^3/d (1.3±0.8, 3.8±0.8 and 5.0±0.2 mgNO_3^--N/L, respectively). The concentration of oxygen entering the reactor was relatively constant (4.14 ±1.8 mg/L), and was below 0.15 mg/L for the two lower flows. For the highest flow (54 m^3/d) $oxygen_{out}$ values varied between 0.5-1.0 mg/L. The NH_4^+- N concentrations in the effluent of the denitrification decreased as the operational flow increased, as also the concentration of PO_4^{3-}-P, sCOD and TP (Table 14). VFA values in the effluent remained constant, independently of operational flow as well as TN. TCOD values in the effluent at the initial flow of 6 m^3/d showed high variation compared to the values obtained for 18 and 54 m^3/d. The pH value of the water

entering the denitrification reactor was 7.5±0.1 at a temperature of 12.5±1.0^{0}C, while the discharge had a pH of 7.3±0.1 and a temperature of 13.5^{0}C ±1.0.

Table 14. IN and OUT values in the SSF and the denitrification reactor at different operational flows (mean ± SD, n=7).

Parameter	**Flows**				**Flows**		
	0.48 m^3/d	**6 m^3/d**	**18 m^3/d**	**54 m^3/d**	**6.48 m^3/d**	**18.48 m^3/d**	**54.48 m^3/d**
	(OUT C_{SSF})	**(IN $C_{DENITRIFICATION}$)**			**(OUT $C_{DENITRIFICATION}$)**		
NO_3^--N	0.0±0.0	5.0±0.2	5.5±0.4	5.8±0.3	1.3±0.8	3.8±0.8	5.0±0.2
NO_2^--N	0.0±0.1	0.1±0.0	0.2±0.0	0.2±0.0	0.2±0.1	0.2±0.1	0.2±0.0
NH_4^+- N (mg/L)	13.1±4.5	1.5±1.8	0.8±0.3	0.8±0.4	4.0±1.3	1.6±0.5	0.9±0.4
PO_4^{3-}-P (mg/L)	3.4±0.5	0.2±0.0	0.2±0.0	0.3±0.0	1.3±0.5	0.5±0.1	0.3±0.0
sCOD (mg/L)	68.6±19.9	8.0±1.2	8.4±5.6	7.7±0.9	13.1±7.4	8.4±5.6	7.6±1.7
VFA_COD (mg/L)	65.4±31.4	0.5±0.5	0.6±0.5	0.8±0.4	0.9±0.2	0.7±0.3	0.9±0.6
TCOD (mg/L)	2943±1427	10±2.4	11.8±4.0	13.5±3.0	20.2±10.0	10.3±2.0	11.3±3.5
TP (mg/L)	104.9±52.3	0.3±0.1	0.7±0.7	0.4±0.0	2.1±0.9	1.0±0.2	0.5±0.08
TN (mg/L)	139.5±84.9	7.1±0.3	8.3±2.7	7.3±0.3	6.3±0.6	6.2±0.1	6.4±0.3

4.6 Mass balance on the SSF

According to the mass balance analysis (Figure 21) the SSF managed to increase the mass of sCOD by 70% and a 14 times increase in the VFA mass entering the denitrification reactor. This represents a constant mass of 20.6 g/d of sCOD and 22.8 g/d of VFA into the reactor. NO_3^--N and NO_2^--N entering the SSF were consumed. Dissolution of 5.9 g/d NH_4^+-N was found in the outlet, corresponding to a 14 times increase in the mass of NH_4^+-N entering the denitrification reactor. In a similar way, 1.1 g/d of PO_4^{3-}-P were discharged from the SSF reactor, corresponding to and increment of 2.6 times folds the mass of PO_4^{3-}-P entering the SSF reactor. The consumption of NOx in the SSF reactor and the production of NH_4^+-N resulted in a net production of 3.3 g N/d as total dissolved nitrogen (TDN).

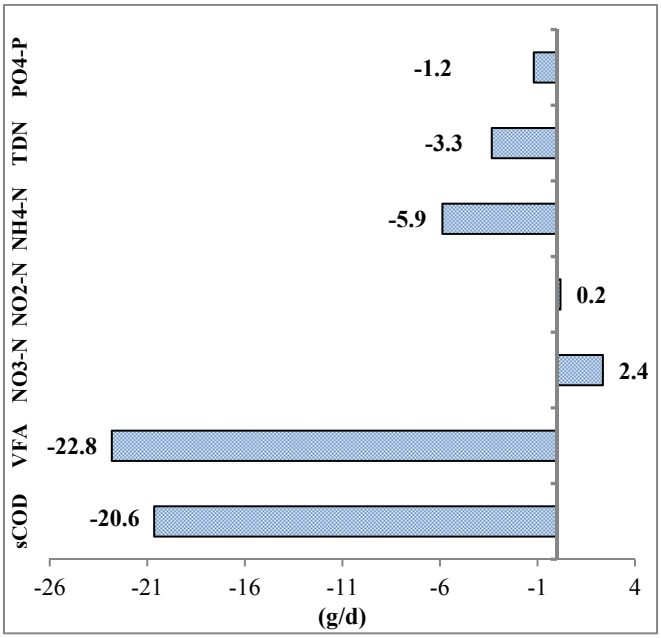


Fig. 21: Mass balance on the SSF, positive (+) values represent consumption while negative (-) values represent formation.

A potential drawback of using waste as internal carbon source is the dissolution of NH_4^+ and PO_4^{3-} (Conroy and Couturier, 2009; Letelier-Gordo et al., 2015). In the present experiment the SSF constantly produced more mass of NH_4^+ and PO_4^{3-} compared to the initial masses measured at day 0 (Table 14).

4.7 Mass balance on the denitrification reactor

The mass balance analyisis showed that the highest removal of NO_3^--N (42.4 g/d) was found at the highest flow (54 m^3/d), corresponding to 13% of the NO_3^--N that entered the reactor (Figure 22). At the lower flows (6 and 18 m^3/d) 25.3 and 24.7 g/d of NO_3^--N were removed respectively which corresponded to 75% and 26% of the NO_3^--N mass that entered the reactor. A 13.2% (52.7 g/d) reduction of TN was found at a flow of 54 m^3/d where 16% (40.6 g/d) and 26% (7.8 g/d) reduction was found for the 6 and 18 m^3/d flows, respectively. The NH_4^+- N mases at the effluent of the denitrification reactor varied according to the operational flow. A 63% mass production (9.9 g NH_4^+- N /d) and 40% production (8.6 g NH_4^+- N /d) were registered at 6 and 18 m^3/d flows, respectively, while 4% (2 g/d g NH_4^+- N /d) was produced at a flow of 54 m^3/d. The removal of NO_3^--N and production of NH_4^+- N balanced the final masses of TDN discharged at the different flows. In this sense, when the denitrification reactor operated at a flow of 54 m^3/d practically three times TDN mass removal was achieved compared to the two previous evaluated flows (Figure 22). Oxygen consumed increased as the operational flows increased, consuming 23.8 gO_2/d (96% consumption), 77.7 g O_2/d (98% consumption) and 181.7 g O_2/d or 86% of total mass of oxygen that entered the reactor, respectively.

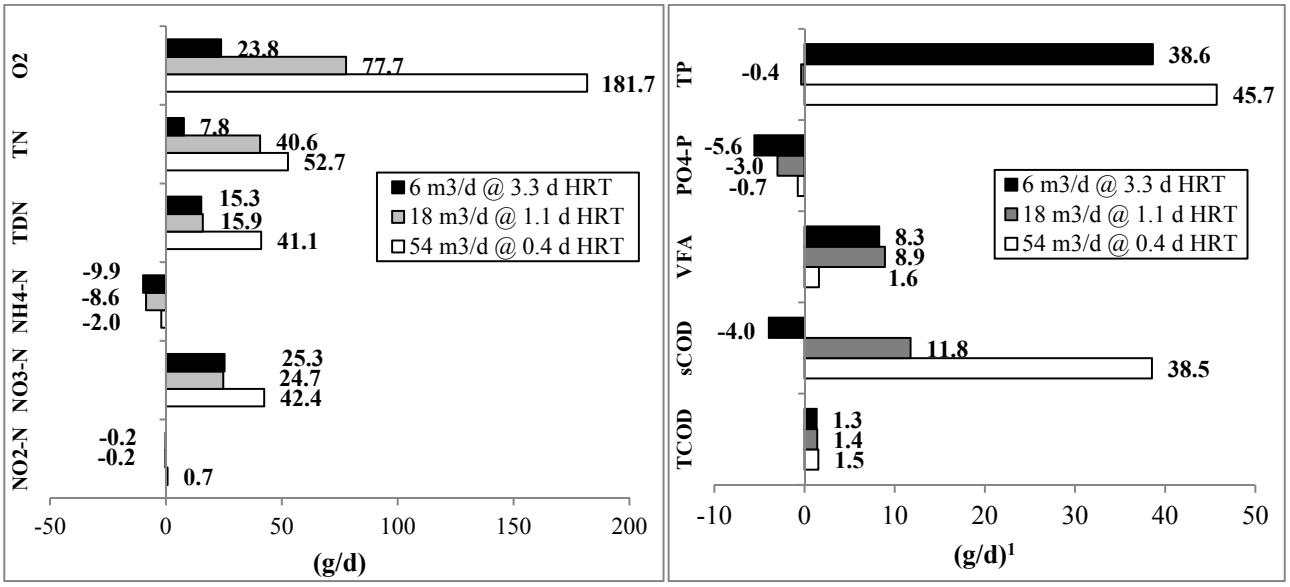


Fig. 22. Mass balance of the single-sludge denitrification reactor, positive (+) values represent consumption while negative (-) values represent formation/accumulation. [1]TCOD values are expressed in Kg/d .

In terms NO_3^--N removal the denitrification reactor did not perform in accordance to the different flows applied. At 6 m^3/d, practically all oxygen entering the reactor was consumed, and an average effluent of 1.3±0.8 mg NO_3^--N/L was measured. Additionally, the mass balance for sCOD showed an accumulation/production of 4 g sCOD/d (5%) and in the case of TCOD a reduction in mass was found but with a high variability of concentration in the effluent (20.2±10 g TCOD/L) (Figure 22). The low flow entering the reactor (HRT 3.3 d) thus resulted in a system operating under substrate (NO_3^-) limited conditions with organic matter being accumulated and degraded inside, and a production of sCOD, NH_4^+ and PO_4^{3-}.At 18 m^3/d, higher removal of NO_3^- was expected since 98% of the oxygen was consumed and anoxic conditions and more substrate (NO_3^-) was available. The results did not reflect these expectations as the amount of NO_3^- removed was similar as found at 6 m^3/d. Probably carbon limitation could be a reasonable explanation for this situation. However, at 54 m^3/d a higher mass of oxygen was removed (181.7 gO_2/d) and although oxic conditions in the effluent were at the limit values for denitrification (0.5-1.0 mg/L) (Henze et al., 2008) double amount of NO_3^- was removed compared to two lower flows. An underlying reason could be unmixed conditions inside the reactor at the intermediate flow (18 m^3/d) creating channeling through the media with the flow more evenly distributed at the higher flow (54 m^3/d) improving the contact between the bacteria and the substrates (organic matter and NO_3^-).

Interestingly, the mass of PO_4^{3-} discharged from the denitrification reactor decreased as the operational flow increased, varying from 5.6 g/d at an operational flow of 6 m^3/d (1.9 times production) to 0.7 g/d (4% production) at 54 m^3/d (Figure 22). Considering that the SSF produced VFAs under anaerobic conditions the removal of PO_4^{3-} might be explained by the activity of polyphosphate accumulating organisms (PAOS) able to accumulate up to 38% of P per amount of bacteria (Henze et al., 2008) or by denitrifiers able to take up phosphorous in excess (up to 11.8% under anoxic or aerobic conditions) (Barak and van Rijn, 2000). Bacterial samples were not analyzed for P content in this study, although the organic particulate fraction obtained in the effluent of the reactor showed between 5-25% of P content exceeding the normal P content of bacteria of 2% (Metcalf and Eddy, 2004). The mass balances showed that NH_4^+ was produced inside the denitrification reactor, presumably as organic matter was degraded. The mass of NH_4^+ produced was similar for the two lower flows (6 and 18 m^3/d) while at 54 m^3/d the mass of NH_4^+ was reduced by 20%. The

unstable anoxic and aerobic conditions found at 54 m^3/d could have promoted some nitrification explaining the reduction in the NH_4^+ masses and concentration found in the effluent.

4.8 Evaluation of implementing a SSF reactor at a low-intensity rainbow trout farm

According to the data obtained for the most optimal flow of the denitrification reactor (54 m^3/d), 1 m^3 of the enhanced sludge in the SSF was able to remove 93.2 g of NO_3^--N plus 379 g of oxygen. In this case study, the trout farm discharged 2160 m^3/d of water with an associated mass of 11.6 Kg NO_3^--N/d and 8.8 Kg O_2/d. To comply with environmental regulations, the farm needs to reduce the actual discharge of TN by 22%, meaning that 2.5 Kg NO_3^--N/d must be removed. To do so, 27 m^3 of organic waste should be treated by the SSF each day, removing in addition 10.3 Kg O_2/d from the effluent to achieve the required anoxic conditions. This is far beyond the amounts of organic waste that the farm can accumulate each day, and use of external carbon sources would probably be required to comply with the environmental regulations. Further improvements could, however, be made to the system to improve the process performance and waste collection, and in this way reduce the cost for external carbon sources.

The improvements may include:

a) Reduce the oxygen concentration in water entering the denitrification reactor.

If oxygen is present in the water (> 1 mg/L), bacteria will always use oxygen over nitrate for metabolic processes (energetically favored) this will influence in the final carbon budget to perform denitrification using endogenous carbon sources. Thus stoichiometrically speaking 0.7 Kg of organic waste expressed as COD is required to remove 1 Kg of O_2 while 2.86 Kg or organic matter expressed as COD are required to reduce 1 Kg of N, meaning that per every Kg of O_2 removed in the affluent approximately a 24% reduction in the denitrification capacity using internal carbon sources is estimated.

b) Apply a flow loop between the effluent and the affluent of the denitrification reactor.

According to the results obtained, not all sCOD and VFAs were consumed in the denitrification reactor, which was probably due to fluid dynamic issues inside the reactor and associated low concentrations affecting the ability of the bacteria to utilize the available carbon substrate. Therefore, recycling the effluent water into the influent of the denitrification reactor would, in theory, increase the usage of the discharged compounds by reducing oxygen masses, and eventually increase the concentration of the substrate, thus leaving more carbon from the SSF to reduce nitrate. Additionally, this configuration would help to reduce the ***otherwise*** increasing concentrations of NH_4^+-N discharged from the denitrification reactor. If 20% of the optimal flow found in this trial (54 m^3/d) is recycled back to the influent of the denitrification reactor, 9.72 g/d of NH_4^+-N could eventually be removed by nitrification. This would result in a consumption of 44 g/d of O_2 simultaneously improving the capacity of the denitrification reactor and reducing the discharge of NH_4^+.

c) Improve the quality of the recovered carbon.

Removing and collecting the organic waste from the raceways in an efficient way, and thus avoiding the constant degradation under aerobic water conditions, will increase the amount of easily degradable organic waste and also give higher sCOD/TCOD yields. In the present case, only 2% of the collected organic matter was transformed into sCOD, whereas values normally range between 20-30% of sCOD from the TCOD. Moreover, a separation of the different organic waste types may be considered. In the present evaluation, all the organic waste derived from the backwash of the drum filter, and the dissolved fractions of the organic waste was therefore lost. This applied specifically to the organic waste coming from the hatchery and sludge

cones, containing a higher fraction of dissolved organic matter and with a better degradability as compared to that coming from biofilter backwash. In this sense, a recommendation would be to discharge all the water from the hatchery and sludge cones directly into the SSF, while applying the drum filter only for treating the organic waste coming from the biofilter backwash.

d) Improve the internal fluid dynamics in the denitrification reactor

The use of media to allow bacterial attachment is a good solution for decoupling the HRT from the bacteria biomass, thus avoiding massive bacterial washout. However, if the media is not correctly mixed, channeling of the flows inside the reactor may occur, especially at low flows. This results in a suboptimal use of the media and an erratic behavior of the reactor. The performance of the denitrification reactor turned out to be flow dependent with the mixing conditions probably affecting the removal capacity. Improvement of the mixing mechanism of the media, or eventually dimensioning the system to operate as plug-flow, would increase the contact time between the substrate and the bacteria especially under low carbon flows.

5. Part IV: Conclusions and future perspectives

The aquaculture industry is considered to be a major future food supplier to a constantly growing population in a world of limited resources. Accomplishing this objective will require the industry to improve its practices and decouple the production from environmental impact. Under a residual resource approach, the main objective of this dissertation was thus to use the organic matter waste produced by the fish and transform it into a new resource in the form of VFA. In this way, organic matter waste becomes an internal carbon resource, reducing the need and associated costs of handling this waste as well as the costs of buying external carbon sources for denitrification. The present thesis developed a methodology and documented how the composition of fish feed affects the type and quantity of carbon compounds produced via hydrolysis and fermentation, and their subsequent potential use as internal carbon source for denitrification. Additionally, the application of a side stream fermenter for enhancing the production of internal carbon from the collected organic waste to perform on-farm denitrification was evaluated on a low-intensity, Danish rainbow trout farm. The major conclusions and future perspectives are:

- A method for estimating the organic matter waste as an internal carbon source allowed for characterization of the hydrolysis and fermentation as two separate processes, and proved to be an accurate approach to analyze the influence of the dietary composition on different process parameters such as carbon yields, nutrient dissolutions and denitrification potential.

- The composition of readily available carbon sources produced by fermentation of fish faeces was qualitatively and quantitatively affected by the dietary composition and the protein source, consequently affecting the denitrification potential. Future perspectives should focus on how the different types of carbon sources affect denitrification process parameters such as denitrification rates, required C:N, biomass production and the best reactor types for this purpose. As the feed composition and the digestibility thereof affect the RAC produced and thus the capacity for using internal carbon sources for biological waste treatment, advantages can be taken by predicting the biological waste treatment potential according to the feed type or improve the feed composition for achieving higher waste treatment potentials.

- The speed of producing soluble carbon sources can be increased by manipulating temperature and a constant pH of 7. This translates into a reduction of the required HRT for the process and by this the required reactor volume and associated costs. None of the treatments evaluated (pH, temperature, enzyme and reactor type) managed to increase the dissolution degree to more than 20-30% therefore further investigations are needed to obtain the maximum potential from the organic waste to produce VFA. Future activities should focus on identifying the parameters limiting the degradation capacity. The organic waste from aquaculture have good potentials for methanogenesis, therefore efforts should be made on how to mimic the conditions of anaerobic digestion with a simultaneous accumulation of VFA. Nutrient media composition, bacterial consortia, reactor configuration and aerobic/anaerobic conditions would be the next steps to follow in this research.

- The use of a SSF showed to increase the dissolved fractions from the obtained organic waste while decoupling the HRT from the denitrification reactor. In low intensity systems such as a brood stock farm the quality of the collected organic waste limits the performance of the system since a big fraction of carbon is already degraded in the system before being recovered for VFA production. Improvements of

the system should focus on reducing the mass of oxygen entering the denitrification reactor, adopting recycling flows and improving organic waste collection methods. In terms of denitrification reactors, the contact between the bacteria and the substrate is primordial for taking full advantage of the process since channeling reduces the efficiency of the system. Improved systems design at an industrial scale and the evaluation of other reactor types would be required for a cost effective alternative in this specific type of farm.

- On larger, more intensive farms having efficient waste removal in operation already the set-up seems to be a promising way for further reducing the nitrogen discharge. Larger Danish farms are interested and hopefully commercial operation of SSF and denitrification reactors in large scale will be in operation in the coming years.

References

Aboutboul, Y., Arviv, R., van Rijn, J., 1995. Anaerobic treatment of intensive fish culture effluents:volatile fatty acid mediated denitrification. Aquaculture 133, 21-32

Amirkolaie, A. K., Leenhouwers, J. I., Verreth, J. A., Schrama, J., 2005. Type of dietary fibre (soluble versus insoluble) influences digestion, faeces characteristics and faecal waste production in Nile tilapia (Orechromis niloticus L.). Aquaculture Research 36, 1157-1166.

Arbiv, R., van Rijn, J., 1995. Performance of a treatment system for inorganic nitrogen removal in intensive aquaculture system. Aquacultural Engineering 14, 189-203.

Batstone, D.J., Keller, J., Angelidaki, I., Kalyuzhnyi, S.V., Pavlostathis, S.G., Rozzi, A., Sanders, W.T.M., Siegrist, H., Vvili, V.A., 2002. The IWA Anaerobic digestion model No 1 (ADM1). Water Science and Technology 45 (10), 65-73.

Balderston, W.L., Sieburth, J.M., 1976. Nitrate removal in closed-system aquaculture by columnar denitrification. Applied and Environmental Microbiology 32: 808-818.

Boley, A., Muller, W.R., Haider, G., 2000. Biodegradable polymers as a solid substrate and biofilm carrier for denitrification in recirculated aquaculture systems. Aquacultural Engineering 22, 75-85

Boopathy, R., Bonvillain, C., Fontenot, Q., Kilgen, M., 2006. Biological treatment of low salinity shrimp aquaculture wastewater using sequencing batch reactor. International Biodeterioration & biodegradation 59, 16-19.

Brinker, A., Reiter, R., 2011. Fish meal replacement by plant protein substitution and guar gum addition in trout feed, part I: effects on feed utilization and fish quality. Aquaculture 310, 350-360.

Chen, Y., Jiang, S., Yuan, H., Zhou, Q., Gu, G., 2007. Hydrolysis and acidification of waste sludge at different pHs. Water Research 41, 683-689.

Cho, C.Y., Hynes, J.D., Wood, K.R., Yoshida, H.K., 1994. Development of high-nutrient-dense, low pollution diets and prediction of aquaculture wastes using biological approaches. Aquaculture 124, 293-305.

Cho, C.Y., Bureau, D. P., 2001. A review of diet formulation strategies and feeding systems to reduce excretory and feed wastes in aquaculture. Aquaculture Research 32, 349 -360.

Christ, O., Wilderer, P.A., Angerhöfer, R., Faulstich, M., 2000. Mathematical modelling of the hydrolysis of anaerobic processes. Water Science and Technology 41, 61 – 65.

Christianson, L., Lepin, C., Tsukuda, S., Saito, K., 2015. Nitrate removal effectiveness of fluidized sulfur-based autotrophic denitrification biofilters for recirculating aquaculture systems. Aquacultural Engineering 68, 10-18.

Cokgor, E. U., Oktay, S., Tas, D. O., Zengin, G. E., Orhon, D., 2008. Influence of pH and temperature on soluble substrate generation with primary sludge fermentation. Bioresource Technology 100, 380 – 386.

Collins, S.A., Øverland, M., Skrede, A., Drew, M., 2013. Effect of plant protein sources on growth rate in salmonids: Meta-analysis of dietary inclusion of soybean, pea, canola/rapeseed meals and protein concetrates. Aquaculture 400, 85-100.

Conroy, J., Couturier, M., 2010. Dissolution of minerals during hydrolysis of fish waste solids. Aquaculture 298 (3), 220–225.

Constantine, H., Fick, M., 1997. Influence of C-sources on the denitrification rate of high-nitrate concentrated industrial wastewater. Water Research 31 (3), 583-589.

Dalsgaard, J., Pedersen, P.B., 2011. Solid and suspended/dissolved waste (N, P, O) from rainbow trout (Oncorhynchus mykiss). Aquaculture 313, 92–99.

Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K., Pedersen, P.B., 2013. Farming different species in RAS in Nordic countries: Current status and future perspectives. Aquacultural Engineering 53, 2-13.

Danish Ministry of Environment, 2012. Bekendtgørelse om miljøgodkendelse og samtidig sagsbehandling af ferskvandsdambrug (Executive order on the environmental approval and handling of freshwater aquaculture systems). Lovtidende A nr. 130. Miljøstyrelsen, Miljøministeriet, Denmark (in Danish).

Dague, R. R., Habben, C. E., Pidaparti, S. R., 1992. Initial studies on the anaerobic sequencing batch reactor. Water Science and Technology 26, 2429-2432.

Diaz, V., Ibanez, R., Gomez, P., Urtiaga, A.M., Ortiz, I., 2012. Kinetics of nitrogen compounds in a commercial marine recirculating aquaculture system. Aquacultural Engineering 50, 20-27.

Eastman, J.A., Ferguson, J.F., 1981. Solubilization of particulate organic carbon during the acid phase of anaerobic digestion. Journal Water pollution Control Federation 53 (3), 266-352.

Elefsiniotis, P., Li, D., 2006. The effect of temperature and carbon source on denitrification using volatile fatty acids. Biochemical Engineer Journal 28, 148-155.

Elefsiniotis, P., Wareham, D.G., 2007. Utilization patterns of volatile fatty acids in the denitrification reaction. Enzyme and Microbial Technology 41, 92-97.

Fass, S., Ganaye, V., Urbain, V., Manem, J., Block, J.C., 1994. Volatile fatty acids as organic carbon sources in denitrification. Environmental Technology 15 (5), 459-467.

Gerardi, M., 2006. Wastewater bacteria. A John Wiley & Sons. New Jersey 188 pp.

Glavic, P., Lukman, R., 2007. Review of sustainability terms and their definitions. Journal of Cleaner Production 15, 1875-1885.

Green, J.A., Hardy, R.W., 2008. The effects of dietary protein:energy ratio and amino acid pattern on nitrogen utilization and excretion of rainbow trout Oncorhynchus mykiss (Walbaum). Journal of Fish Biology 73, 663-682.

Gutierrez, M.T., Malone, R., Rusch, K., 2011. Evaluation of polyhydroxubutyrate as a carbon source for recirculating aquaculture water denitrification. Aquacultural Engineering 51, 36-43.

Hamlin, H.J., Michaels, J.T., Beaulaton, C.M., Graham, W.F., Dutt, W., Steinbach, P., Losordo, T.M., Schrader, K.K., Main, K.L., 2008. Comparing denitrification rates and carbon sources in commercial scale upflow denitrification filters in aquaculture. Aquacultural Engineering 38, 79-92.

Henze, M., 1991. Capabilities of biological nitrogen removal processes from wastewater. Water Science and Technology 23 (4-6), 669-679.

Henze, M., Harremoës, P., Jansen, J.l.C., Arvin, E., 2002. Wastewater treatment. Biological and chemical process. Springer 3rd edition. 430 pp.

Henze, M., van Loosdrecht, M., Ekama, G. A., Brjanovic, D., 2008. Biological Wastewater Treatment: principles, modelling and design. IWA Publishing 2008, 511 pp.

Her, J., Huang, J., 1995. Influences of carbon source and C/N ratio on nitrate/nitrite denitrification and carbon breakthrough. Bioresource Technology 54, 45-51.

Hidalgo, M. C., Urea, E., Sanz, A., 1998. Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. Aquaculture 170, 267-283.

Huiliñir, C., Hernandez, S., Aspe, E., Roeckel, M., 2012. Simultaneous nitrate organic matter removal from salmon industry wastewater: The effect of C/N ratio, nitrate concentration and organic load rate on batch continuous process. Journal of Environmental Management 101, 82 – 91.

Jewell, W.J., Cummings, R.J., 1990. Expanded bed treatment of complete recycle aquaculture systems. Water Science and Technology 22, 443-450.

Jokumsen, A Svendsen, L.,. 2010. Farming of Freshwater Rainbow Trout in Denmark. DTU Aqua, National Institute of Aquatic Resources. Report no 219-2010, DK. 47 pp.

Klas, S., Mozes, N., Lahav, O., 2006. A conceptual, Stoichiometry-based model for single-sludge denitrification in recirculating aquaculture systems. Aquaculture 259, 328 – 341.

Lee, N., Welander, T., 1996. The effect of different carbon sources on respiratory denitrification in biological waste water. Journal of Fermentation and Bioengineering 83 (3), 277-285.

Martins, C.I.M., Eding, E.H., Verdegem, M.C.J., HeinSSFoek, L.T.N., Schneider, O., Blancheton, J, E., Roque d'Orbcasteld, J., Verreth, A.J., 2010. New developments in recirculating aquaculture systems in Europe: a perspective on environmental sustainability. Aquacultural Engineering 43, 83–93.

Mc Carty, P.L., Smith, D.P., 1986. Anaerobic wastewater treatment. Environmental Science and Technology. 20(12), 1200-1206.

Meriac, A., Edinng, Ep, H., Kamstra, A., Busscher, J., Schrama, J, W., Johan, A. H., 2014. Denitrification on internal carbon sources in RAS is limited by fibers in fecal waste of rainbow trout. Aquaculture 434, 264-271.

Metcalf and Eddy. Wastewater Engineering, Treatment and Reuse, 4th Ed. Mc Graw Hill, 2004.

Mirsoyan, N., Tal, Y., Gross, A., 2010. Anaerobic digestión of sludge from intensive recirculating aquacutlure systems:Review. Aquaculture 306, 1-6.

Mirsoyan, N., Gross, A., 2013. Use of UASB reactors for brackish aquaculture sludge digestion under different conditions. Water research 47, 2843-2850.

Morgenroth, E., Kommedal, R., Harremoes, P., 2002. Processes and modelling of hydrolysis of particulate organic matter in anaerobic wastewater treatment – a review. Water Science and Technology 45, 25-40.

Muyzer, G., Stams, A. J. M., 2008. The ecology and biotechnology of sulphate-reducing bacteria. Nature reviews microbiology 6, 441-454.

Ni, B.J. Ruscalleda, M., Pellicer-Nacher, C., Smets, B. F., 2011. Modeling Nitrous oxide production during biological nitrogen removal via nitrification and denitrification: Extension to the general ASM models. Environmental Science and Technology 45, 7768-7776.

Nijhof, M., 1994. Theoretical effects of feed composition feed conversion and feed spillage on waste discharge in fish culture. Journal of Applied Ichthyology 10, 274-283.

Otte, G., Rosenthal, H., 1979. Management of a closed brackish water system for high density fish culture by biological and chemical water treatment. Aquaculture 18, 169-181.

Parmar, N., Singh, A., Ward, O.P., 2001. Enzyme treatment to reduce solids and improive settling of sewage sludge. Journal of Industrial Microbiology & Biotechnology 26, 382-386.

Pavlostathis, S.G., Giraldo-Gomez, E., 1991. Kinetics of anaerobic treatment. Water Science and Technology 24, 35-59.

Pratoomyot, J., Bendiksen, E.A., Bell, J. G., Tocher, D. R., 2010. Effects of increasing replacement of dietary fishmeal with plant protein sources on growth performance and body lipid composition of Atlantic salmon (*Salmo salar* L.). Aquaculture 305, 124-132.

Rajagopal, R., Beline, F., 2011. Nitrogen removal via nitrite pathway and the related nitrous oxide during piggery wastewater treatment. Bioresource Technology 102, 4042-4046.

Roman, H.J., Burguess, J.E., Pletschke, B.I., 2006. Enzyme treatment to decrease solids and improve digestion of primary sewage sludge. African Journal of Biotechnology 5, 963-967.

Saliling, W.J.B., Westerman, P.W., Losordo, T.M., 2007. Wood chips and wheat straw as alternative biofilter media for denitrification reactors treating aquaculture and other wastewaters with high nitrate concentrations. 2007. Aquacultural Engineering 37, 222-233.

Suheyl, A., Henze, M., 2008. Biological hydrolysis and acidification of sludge under anaerobic conditions: The effect of sludge type and origin on the production and composition of volatile fatty acids. Water Research 42, 3729-3738.

Suhr, K.I., Pedersen, P.B., Arvin, E., 2012. End-of-pipe denitrification using RAS effluent waste streams: Effect of C/N-ratio and hydraulic retention time. Aquacultural Engineering 53, 57-64.

Suhr, K.I., Pedersen, L.-F., Nielsen, J.L., 2014. End-of-pipe single-sludge denitrification in pilot-scale recirculating aquaculture systems. Aquacultural engineering 62, 28-35.

Suzuki, Y., Maruyama, T., Numate, H., Sato, H., Asakawa, M., 2003. Performance of a closed recirculating system with foam separation, nitrification and denitrification units for intensive culture of eel: towards zero emission. Aquacultural Engineer 29, 165-182.

Tal, Y., Schreier, H., Sowers, K.R., Stubblefield, J., Place, A., Zohar, Y., 2009. Environmentally sustainable land-based marine aquaculture. Aquaculture 286, 28-35.

Talbot, C., Hole, R., 1994. Fish diets and the control of eutrophication resulting from aquaculture. Journal of Applied Ichthyology 10, 258-270.

Tiedje, J.M., 1990. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. P. 179-244. *In* A.J.B Zehnder (ed), Environmetal microbiology of anaerobes. John Wiley and Sons, N.Y. pp .

Timmons, M., Ebeling, J., Piedrahita, R., 2009. Acuicultura en Sistemas de Recirculación. First Edition in Spanish. Editorial CayugaAquaVentures, LLC. Ithaca, NY. 959 pp.

Tsukuda, S., Christianson, L., Kolb, A., Saito, K., Summerfelt, S., 2015. Heterotrophic denitrification of aquaculture effluent using fludized sand biofilters. Aquacultural Engineering 64, 49-59.

Ucisik, A., Henze, M., 2008. Biological hydrolysis and acidification of sludge under anaerobic conditions: The effect of sludge type and origin on the production and composition of volatile fatty acids. Water Research 42, 3729-3738.

van Rijn, J., Rivera, G., 1990. Aerobic and anaerobic biofiltration in an aquaculture unit-nitrite accumulation as results of nitrification and denitrification. Aquacultural Engineering 94, 217-234.

van Rijn, J., Fonarev, N., Berkowitz, B., 1995. Anaerobic treatment of intensive fish culture effluents: digestion of fish feed and release of volatile fatty acids. Aquaculture 133 (1), 9–20.

van Rijn, J., Tal, Y., Schreier, H.J., 2006. Denitrification in recirculating systems: theory and application. Aquacultural Engineering 34 (3), 364-376.

van Rijn, J., 2013. Waste treatment in recirculating aquaculture systems. Aquacultural Engineering 53, 49-56.

Vavilin. V. A., Fernandez., Palatsi, J., Flotats, X., 2008. Hydrolysis kinetics in anaerobic degradation of particulate organic material: An overview. Waste Management 28, 939-951.

Von Ahnen, M., Pedersen, P.B., Daalsgard, J., 2016. Start-up performance of a woodchip bioreactor operated end-of-pipe at a commercial fish farm-A case study. Aquacultural Engineering 74, 96-104.

Yatong, X., 1996. Volatile fatty acids carbon source for biological denitrification. Journal Environmental Science 8, 257-268.

Yuan, Q., Sparling, R., Oleszkiewicz, J.A., 2011. VFA generation from waste activated sludge: Effect of temperature and mixing. Chemosphere 82, 603-607.

Wolf, I. A., Wasserman, A. E., 1972. Nitrates, Nitrites and Nitrosamines. Science 177, 4030.

Wu, H., Yang, D., Zhou, Q., Song, Z., 2009. The effect of pH on anaerobic fermentation of primary sludge at room temperature. Journal of Hazardous Materials 172, 196-201.

Appendix

Paper I: Reducing dietary protein:energy (P:E) ratio changes solubilization and fermentation of rainbow trout (*Oncorhynchus mykiss*) faeces.

Paper II: The composition of readily available carbon sources produced by fermentation of fish faeces is affected by dietary protein:energy ratios.

Paper III (manuscript): Soybean meal in rainbow trout (*Oncorhynchus mykiss*) feed: Effects on nutrient utilization, waste production, and waste treatment potential

Paper IV (manuscript): Applicability of internal carbon sources for a denitrification system on a Danish brood stock farm, a mass balance approach.

Paper I

Aquacultural Engineering 66 (2015) 22–29

Contents lists available at ScienceDirect

Aquacultural Engineering

journal homepage: www.elsevier.com/locate/aqua-online

Reducing the dietary protein:energy (P:E) ratio changes solubilization and fermentation of rainbow trout (*Oncorhynchus mykiss*) faeces

Carlos O. Letelier-Gordo[a,*], Johanne Dalsgaard[a], Karin I. Suhr[a], Kim Schøn Ekmann[b], Per Bovbjerg Pedersen[a]

[a] Technical University of Denmark, DTU Aqua, Section for Aquaculture, The North Sea Research Centre, P.O. Box 101, DK-9850 Hirtshals, Denmark
[b] BioMar A/S, Myl. Erichsensvej 35, DK-7330 Brande, Denmark

ARTICLE INFO

Article history:
Received 14 November 2014
Accepted 18 February 2015
Available online 26 February 2015

Keywords:
Protein:energy
Hydrolysis
Solubilization
Fermentation
VFA
C:N

ABSTRACT

Nutrients discharged from aquaculture industries can detrimentally affect water recipients, and this problem must be addressed if the production is to be decoupled from the natural environment. Denitrification is a process by which nitrate is removed using soluble, readily biodegradable carbon compounds. Hydrolysis and concomitant fermentation of organic solids produces such soluble carbon compounds e.g. in the form of volatile fatty acids (VFAs). The current study examined the hydrolysis and the production of VFAs, the carbon:nitrogen ratio (C:N), and the release of nutrients (phosphorus and ammonium) from hydrolyzing and fermenting settable faecal solids (SFS) obtained from rainbow trout (*Oncorhynchus mykiss*). Triplicate tanks of fish were fed five isoenergetic experimental diets with different protein:energy (P:E) ratios: 15, 17, 19, 21, and 23. The SFS from four consecutive days were collected and pooled prior to incubation in 15, 1 L anoxic/anaerobic batch reactors maintained at $20 \pm 2\,^{\circ}C$ and continuous magnetic stirring. Daily samples from the batch reactors were obtained for 7 successive days and analyzed for total ammonia nitrogen (TAN), phosphorus expressed as orthophosphate (PO_4^{3-}-P), VFA, and soluble COD (sCOD). The results showed that the two lowest P:E ratio diets (i.e. 15 and 17) produced SFS with a significantly higher degree of solubilization measured as sCOD:total chemical oxygen demand (TCOD), compared to the higher P:E ratio diet 21 (0.30–0.29 versus 0.24 g sCOD/g TCOD). Inversely, SFS deriving from the lowest P:E ratio diet (i.e. 15) displayed the lowest degree of fermentation measured as VFAs/sCOD, compared to SFS deriving from the four higher P:E diets (0.36 versus 0.51–0.56 g VFA/g sCOD). In the same way, the lowest P:E diet showed a significantly lower solubilization of nitrogen measured as TAN:total Kjeldahl Nitrogen (TKN) compared to the three highest P:E diets (i.e. 19–23; 0.14 versus 0.26–0.34 g TAN/g TKN). The two lowest P:E diets (i.e. 15–17) showed on the contrary the highest solubilization of phosphorus expressed as PO_4^{3-}-P/total phosphorus (TP) (0.15 and 0.08 g/g, respectively) probably due to the lower pH obtained. All SFS produced enough soluble carbon, measured as VFAs, to stoichiometrically denitrify the nitrogen (N) contained in the faeces and potentially additionally 86–100% of all N produced from the fish culture process.

1. Introduction

Optimizing environmental sustainability in aquaculture requires a reduction in nutrient and organic discharges to aquatic systems (i.e. river and lakes), ensuring that these systems are not outpaced from their intrinsic carrying capacity. To achieve environmental sustainability in aquaculture, authorities in many countries have implemented strict regulations such as e.g. in Denmark, where nutrient discharge from fish farms are regulated by nutrient discharge quotas (Danish Ministry of Environment, 2012). The application of a strict regulation has affected the growth of the sector, causing stagnation of freshwater aquaculture production in Scandinavia as compared to the growing aquaculture tendency on a global level (FAO, 2012; Dalsgaard et al., 2013). On the other hand, the policy applied has forced the sector to improve their practices and water treatment technologies, progressively becoming more competitive and environmentally sustainable. The concept of end-of-pipe treatment (EOP; Glavic and Lukman, 2007), which refers to the practice of treating polluting substances at the end of the production process, is thus becoming increasingly relevant as the industry seeks to obtain environmentally sustainable growth.

* Corresponding author. Tel.: +45 35883299.
E-mail address: colg@aqua.dtu.dk (C.O. Letelier-Gordo).

http://dx.doi.org/10.1016/j.aquaeng.2015.02.002
0144-8609/

In recirculating aquaculture systems (RAS) where nitrification has already been applied within the system and nitrate is the main nitrogenous product, it makes sense to posteriorly include microbial denitrification to reduce the amount of total nitrogen being discharged. The process has been applied worldwide in wastewater treatment, where the efficiency by which nitrate is removed depends on the availability of easily degradable carbon sources, often limiting the process and affecting the associated operational costs (Henze et al., 2002; Metcalf and Eddy, 2004; Suheyl and Henze, 2008). Similarly, the limited availability of soluble, readily available carbon sources in aquaculture effluents has been a matter of discussion with respect to applying denitrification as an in-line configuration or as EOP treatment (Klas et al., 2006; Suhr et al., 2013). To overcome this limitation, the use of external carbon sources such as methanol, acetic acid or dextrose have frequently been used (Hamlin et al., 2008; Huiliñir et al., 2012).

Organic matter and nitrate are removed during the denitrification process, and the carbon:nitrogen (C:N) ratio is a conditional parameter that expresses the plausibility for denitrification to occur. The type of carbon source employed dictates the required C:N ratio (Henze et al., 2002; Metcalf and Eddy, 2004). Henze et al. (2002) has for example proposed different C:N ratios according to the type of organic matter from wastewater employed as carbon sources, advising 4–5 kg COD/kg N or 2.9–3.5 kg acetic acid/kg N.

To save operational costs in RAS, internal carbon sources originating from fish faeces might be used for denitrification as originally demonstrated by Jewell and Cummings (1990). To optimize the usability of faeces for this purpose, the amount of soluble, biodegradable carbon sources present as for example volatile fatty acids (VFA) must be maximized (Henze et al., 2002). This can be achieved by letting the settleable faecal solids (SFS) produced by fish undergo hydrolysis and concomitant fermentation. During the hydrolysis process, a series of hydrolytic and facultative anaerobic bacteria convert complex substrates (carbohydrates, lipids, and proteins) into simple organic compounds (sugars, fatty acids, and amino acids). In the concurrent fermentation process, these simple organic compounds are assimilated by facultative anaerobic bacteria, resulting in the production of carbon dioxide, hydrogen gas, alcohols, and organic acids in the form of VFA (Gerardi, 2006).

Dietary nutrients that have not been assimilated by the cultured organism are the major source of waste produced in intensive aquaculture systems (Cho et al., 1994; Timmons et al., 2009). Consequently, the feed composition, its inherent properties, and the associated digestibility and nutrient utilization will dictate the waste properties, and the waste output masses and chemistry in the effluents (Nijhof, 1994; Cho and Bureau, 2001; Amirkolaie, 2011).

It has previously been reported that a reduction in the ratio of dietary digestible protein to digestible energy (DP:DE) can reduce the nitrogen waste output (Cho and Bureau, 2001; Green and Hardy, 2008). In the case of phosphorus, the waste outputs can be minimized by optimizing the utilization of dietary phosphorus (Bureau and Cho, 1999; Coloso et al., 2003; Dalsgaard et al., 2009). Therefore, since the diet formulation and related apparent digestibility and nutrient utilization has a direct effect on the effluent characteristics, it should in theory be possible to explicitly use or eventually develop feed that will produce/result in specific SFS that can sustain or enhance biological nutrient removal. Important here is the mass and quality of the produced carbon sources available for pursuing single-sludge denitrification (i.e. supplying the electron donor from waste produced within the system) (Klas et al., 2006), and to some extent the mass of nitrogen and phosphorus released to the bulk phase.

With this approach in mind, the purpose of the present study was to investigate the impact of different dietary protein:energy (P:E) ratios on the hydrolysis and fermentation processes in SFS produced by juvenile rainbow trout (*Oncorhynchus mykiss*). Special emphasis was given to the dynamic production of soluble COD (sCOD), soluble readily biodegradable carbon sources (VFAs), and nutrient release dynamics; total ammonia nitrogen (TAN) and phosphorus expressed as orthophosphate (PO_4^{3-}-P), as well as the obtained and potential C:N ratios which are pertinent for obtaining maximized denitrification.

2. Materials and methods

2.1. Fish and experimental diets

An experiment to investigate the effects of different dietary P:E ratios on the hydrolysis and fermentation processes of collected SFS from juvenile rainbow trout was carried out at DTU Aqua's research facility in Hirtshals, Denmark. A randomized, single-factor experiment was performed using five isoenergetic experimental diets (P:E 15, 17, 19, 21, 23) with different levels of protein:energy, and three replicate tanks ($n = 3$) for each diet. The experimental diets were formulated and produced by Biomar A/S, Denmark applying a twin screw extruder (Clextral BC-45, Clextral S.A., Firminy, France) to extrude the feed as 3 mm pellets. Ingredients and analyzed proximate composition of the diets are shown in Table 1.

The fish were maintained in 15 separate, flow-through tanks in a nutrient mass balance system (NMBS) as described in Dalsgaard and Pedersen (2011). Each individual 189 L tank was stocked with approximately 1.3 kg fish (initial mean weight 56.2 ± 6 g), and was maintained at 12.3 ± 0.3 °C, an inflow of 40 L/h/tank (municipal tap water), and a 15 h light: 9 h dark photoperiod.

The fish were acclimatized to feed type and rearing conditions for 7 days followed by a period of 9 days to establish fish performance. The fish were subsequently fed a fixed amount of feed once a day (10:00 AM), corresponding to 1.6% of the estimated biomass in each tank, in order to collect SFS from a well-defined amount of feed for 4 consecutive days.

2.2. Settleable faecal solids (SFS) collection

The produced SFS from each tank were collected daily (24 h) from sedimentation columns (40 mm diameter collectors) mounted at the bottom cones of the NMBS via a union valve. The collectors were enclosed in styrofoam containers with water and ice to maintain the collected SFS at 0 °C. The union valve was closed during feeding to prevent feed waste from entering the collectors, and any uneaten pellets were immediately removed after the daily

Table 1
Ingredients used and analyzed gross composition of the five experimental diets.

Ingredients (%)	P:E_15	P:E_17	P:E_19	P:E_21	P:E_23
Fish meal[a]	42.9	50.8	58.7	66.5	74.4
Wheat	37.3	30.4	23.6	16.7	9.8
Fish oil	21.2	19.9	18.6	17.3	16.0
Vitamins and minerals[b]	0.30	0.30	0.30	0.30	0.30
Proximate composition (%)[c]					
Dry matter	93.4	93.5	93.7	94.8	96.6
Protein	32.7	37.2	42.5	46.9	50.2
Lipid	27.2	26.4	25.9	25.0	24.5
Ash	7.55	8.71	9.86	11.0	11.2
Phosphorous	1.3	1.5	1.7	1.9	1.9
NFE (nitrogen free extracts)[d]	24.6	19.7	13.7	10.0	8.8
Gross energy (Kj/g)[e]	22.5	22.3	22.1	22.0	22.4

[a] SA 68 superprime Perú, South America (68% protein).
[b] Premix Dk 3.
[c] Proximate composition analyzed as described in Dalsgaard and Pedersen (2011).
[d] NFE calculated as: dry matter–protein–lipid–ash.
[e] Gross energy measured using a bomb calorimeter (IKA-Calorimeter C7000, IKA Analystechnik, Heitersheim, Germany).

feeding period and counted to derive the feed intake. The collected SFS were pooled for 4 consecutive days and stored throughout at 0 °C to minimize potential degradation.

2.3. Hydrolysis/fermentation trial

The four days pooled SFS from each of the 15 tanks were transferred to fifteen 1 L enclosed Blue Cap bottles (SCHOTT Duran, Germany) serving as anoxic/anaerobic batch reactors. The reactors were kept at room temperature (20 ± 2 °C) with continuous magnetic stirring at 200 rpm (Big Squid, IKA, Germany). The bottles were sealed with screw caps with two ports for sampling purposes (cap_GL, Duran Group, Germany), designed to avoid potential oxygen interference. Nitrogen gas was purged for 5 min into each bottle to ensure equal anoxic/anaerobic conditions in each batch before starting the hydrolysis/fermentation trial. The trial was terminated after 7 days to avoid methanogenic activity (Miron et al., 1998). Daily samples of 30 mL were obtained for analyses of TAN, dissolved phosphorous (PO_4^{3-}-P), VFA, and soluble COD (sCOD) using a 20 mL syringe. At the same time, pH and temperature were monitored using a portable meter (Hach HQ40d, Hach Lange, Germany). Total solids (TS), total volatile solids (TVS), total COD (TCOD), total phosphorus (TP), and total Kjeldahl Nitrogen (TKN) were analyzed in the SFS at the start of the hydrolysis/fermentation period (day 0).

2.4. Analytical methods

The daily samples were centrifuged immediately (4500 rpm for 15 min at 0 °C), and subsequently filtrated through 0.2 μm Filtropour S syringe filters (SARSTEDT, Germany). Analyses of VFA were carried out the same day using a test kit procedure (LCK 365, Hach Lange, Germany). Sulfuric acid was added to the rest of each filtrated sample for preservation purposes and subsequent analysis. Furthermore, preserved subsamples for TP, TKN, and TCOD analyses were frozen and analyzed later. Total ammonia nitrogen was determined using indophenolblue with salicylate (DS/EN 224, 1975), dissolved phosphorus was determined according to the ammonium molybdate spectrometric method and expressed as PO_4^{3-}-P (ISO 6878, 2004), and sCOD was determined using digestion vials (LCK 014, Hach Lange, Germany). Total solids (TS) and Total volatile solids (TVS) analysis were performed according to the methodology proposed by Metcalf and Eddy (2004). Total phosphorus was determined according to a spectrometric method (ISO 6491, 1998), TKN by digesting and distilling the samples (ISO 5983-2, 2005), and TCOD by using digestion vials (LCK 914, Hach Lange, Germany). Diets were ground using Krups Speedy Pro homogenizer and SFS samples were thawed and prepared using Ultra Turrax homogenizer before analyzing for TKN (ISO 5983-2, 2005 (crude protein = 6.25 × Kjeldahl N)), lipid (Bligh and Dyer, 1959), total phosphorus (ISO 6491, 1998), totals solids (=dry matter content) and ash (Metcalf and Eddy, 2004). Gross energy in diets was measured using a bomb calorimeter (IKA-Calorimeter C7000, IKA Analysentechnik, Heitersheim, Germany) after drying for 48 h at 60 °C. Nitrogen free extract (NFE) was calculated as: NFE = TS–ash–lipid–protein.

2.5. Data treatment and statistical analysis

The hydrolysis process (degree of solubilization) was expressed as sCOD/TCOD, and the fermentation process (VFA production) was expressed as VFA/sCOD *sensu* Suheyl and Henze (2008). The process related to dissolution of nitrogen was expressed as TAN/TKN, and the dissolution of phosphorus was expressed as PO_4^{3-}-P/TP. The obtained C:N is expressed as the relation between VFA/TN, whereas the potential C:N is the relation between TCOD/TN. For calculating the amount of nitrate that can be removed using the obtained C:N, a ratio of 2.9 was used and in the case of the potential C:N a ratio of 5 was used (Henze et al., 2002). The dissolved N fraction accounted in the potential C:N calculations is estimated according to Dalsgaard and Pedersen (2011) and assuming 1 g NH_4^+-N is oxidized to 1 g NO^-_3-N.

To test for significant differences between obtained values for each defined process, the results of the five different dietary treatments where compared at day 0 and day 7. Statistical analyses were carried out using the R software version 3 (R Core Team, 2013). The characteristics of the SFS (i.e. the content of TS, TVS, TKN, protein, lipid, TP and NFE), and the hydrolysis/fermentation parameters (i.e. sCOD/TCOD, VFA/sCOD, TAN/TKN and PO_4^{3-}-P/TP) derived at day 0 and day 7, were subjected to one-way ANOVA analysis followed by Tukey–Kramer multiple comparison of means test with a 95% family-wise confidence level. Differences were considered significant at $P < 0.05$, and values are stated as the mean ± standard deviation (SD).

3. Results

3.1. Characterization of SFS

A summary of the characteristics of the SFS examined in the hydrolysis/fermentation trial (i.e. at day 0) is presented in Table 2. The TS content of all SFS samples ranged between 0.17 and 0.20 g/g feed consumed, and the TVS content ranged between 0.10 and 0.14 g/g feed consumed. No significant differences between treatment groups were found for TS, while the TVS produced per unit feed consumed was significantly higher in $SFS_{P:E\ 15}$ compared to $SFS_{P:E\ 17}$, $SFS_{P:E\ 19}$, and $SFS_{P:E\ 21}$.

The lipid content ranged between 21.0 and 29.2 mg/g feed consumed with no significant differences between dietary treatment groups. The TKN content ranged between 5.8 and 8.6 mg/g feed consumed, and the calculated protein content ranged between 36.0 and 53.7 mg/g feed consumed. The TKN (and hence protein) content was significantly higher in SFS from fish fed the highest N diets ($SFS_{P:E\ 21}$ and $SFS_{P:E\ 23}$) compared to the lowest N diets ($SFS_{P:E\ 15}$ and $SFS_{P:E\ 17}$). Furthermore, the content was significantly higher in SFS from fish fed the intermediate N diet ($SFS_{P:E\ 19}$) compared to the lowest N diet ($SFS_{P:E\ 15}$) and significantly lower compared to the higher N diet ($SFS_{P:E\ 23}$). The NFE content ranged between 71.8 and 100.7 mg/g feed consumed, being significantly higher in $SFS_{P:E\ 15}$ compared to the rest of the SFS. The content of TP ranged between 7.9 and 11.2 mg/g feed consumed with no significant differences between treatments. Ash values ranged between 49.9 and 76.0 mg/g feed consumed with significant difference between the lower P:E diets ($SFS_{P:E\ 15}$ and $SFS_{P:E\ 17}$), the intermediate P:E diet ($SFS_{P:E\ 19}$), and the higher P:E diets ($SFS_{P:E\ 21}$ and $SFS_{P:E\ 23}$).

3.2. Hydrolysis (degree of solubilization)

The results on the degree of solubilization (sCOD/TCOD) are shown for each consecutive day and feed type in Fig. 1. The sCOD/TCOD yield at day 0 for all SFS ranged between 0.17 and 0.20 g/g, showing no significant difference between feed types. The highest daily increment in the degree of solubilization was found at day 1 with increments ranging from 4.0 to 4.7% for all diets. The solubilization stabilized during the rest of the experiment, with a net increase not exceeding 2.2%/day.

At day 7, the sCOD/TCOD values ranged between 0.24 and 0.30 g/g, and the degree of solubilization in $SFS_{P:E\ 15}$ and $SFS_{P:E\ 17}$ was significantly higher than in $SFS_{P:E\ 21}$. During the 7 days a net production of 1.51 g sCOD was found for $SFS_{P:E\ 21}$ compared to 1.92 and 1.65 g sCOD for $SFS_{P:E\ 17}$ and $SFS_{P:E\ 15}$, respectively.

Table 2
Characteristics (day 0) of settable faecal solids (SFS) produced from the different diets and posteriorly used in the hydrolysis/fermentation batch study (mean ± SD, $n = 3$). Data are expressed as masses produced/feed consumed, and are based on daily sampling and subsequent pooling for four consecutive days[a].

Diet	P:E_15	P:E_17	P:E_19	P:E_21	PE:_23
TS (g/g)	0.19^{a} ± 0.01	0.17^{a} ± 0.01	0.17^{a} ± 0.02	0.18^{a} ± 0.01	0.20^{a} ± 0.01
TVS (g/g)[b]	0.14^{c} ± 0.01	0.11^{ab} ± 0.01	0.10^{ab} ± 0.01	0.10^{ab} ± 0.01	0.12^{ac} ± 0.00
TKN (mgN/g)	5.8^{d} ± 0.4	6.5^{bd} ± 0.4	7.1^{bc} ± 0.8	8.1^{ac} ± 0.4	8.6^{a} ± 0.4
Protein (mg/g)[c]	36.0^{d} ± 2.2	40.8^{bd} ± 4.7	44.6^{bc} ± 4.7	50.6^{ac} ± 2.2	53.7^{a} ± 2.4
Lipid (mg/g)	29.2^{a} ± 3.3	21.0^{a} ± 4.8	21.8^{a} ± 5.6	24.0^{a} ± 6.1	28.1^{a} ± 2.9
NFE (mg/g)[d]	100.7^{b} ± 1.3	84.1 ± 6.4^{a}	73.0 ± 7.7^{a}	71.8 ± 2.8^{a}	84.3 ± 4.2^{a}
TP (mg/g)	7.9^{a} ± 0.9	11.2^{a} ± 2.1	9.6^{a} ± 1.1	9.1^{a} ± 3.1	9.4^{a} ± 2.5
Ash (mg/g)	49.9 ± 4.4^{a}	60.7 ± 4.2^{ac}	66.6 ± 5.3^{ce}	74.0 ± 2.9^{bde}	76.0 ± 2.9^{bde}
Fish performance					
SGR[e]	2.10^{a} ± 0.04	2.27^{ab} ± 0.03	2.30^{abc} ± 0.09	2.52^{bc} ± 0.12	2.55^{c} ± 0.06
FCR[f]	0.82^{a} ± 0.02	0.75^{ab} ± 0.01	0.74^{b} ± 0.03	0.67^{c} ± 0.03	0.66^{c} ± 0.02

[a] Values within rows not sharing a common superscript letter were significantly different (Tukey–Kramer, $P < 0.05$).
[b] TVS: total volatile solids at day 0.
[c] Protein was derived from TKN by multiplying by 6.25.
[d] NFE was calculated as NFE = TS–protein–lipid–ash.
[e] SGR: specific growth rate calculated as $\mathrm{Ln}(W(t_i)/W(t_0))/(t_i - t_0) \times 100$, $W(t_i)$ and $W(t_0)$ being the biomass at the end (t_i) and start (t_0) of the growth evaluation period (9 days).
[f] FCR: feed conversion ratio calculated as feed consumed ($t_i - t_0$)/biomass gain ($t_i - t_0$).

3.3. VFA production (degree of fermentation)

The results on the degree of fermentation (VFA/sCOD) are shown for each day and feed type in Fig. 2. At day 0 the values for all the SFS were between 0.09 and 0.13 g VFA/g sCOD, showing no significant difference between them. The VFA/sCOD ratio increased by 19.8–24.9% from day 0 to 1 for all dietary treatment groups, whereas a net increment of less than 8%/day was obtained towards the end of the evaluation (i.e. day 6 and 7). Day 7 values for all the SFS varied between 0.36 and 0.57 g VFA/g sCOD, having significantly lower yields for $SFS_{P:E\ 15}$ (1.84 g VFA) than for the rest of the SFS producing a total VFA mass between 1.98 and 2.57 g.

Relating VFA production to the amount of feed consumed, the values ranged between 3.61 and 4.74 mg/g on day 0, increasing to 23.02–29.43 mg/g at day 7 (Fig. 3). No significant differences between treatment groups were found at day 0 or day 7.

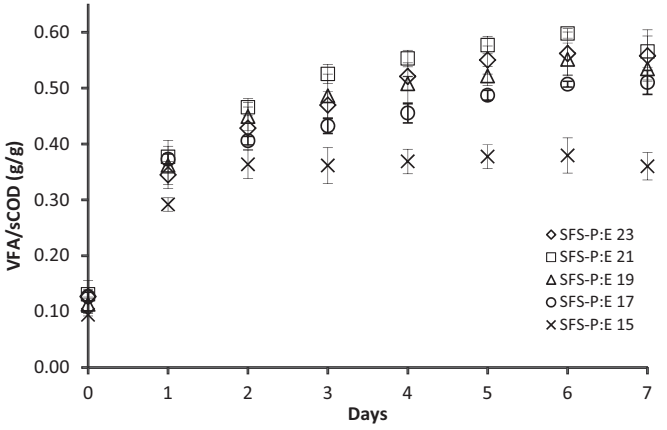


Fig. 2. Cumulative VFA production (degree of fermentation) shown as VFA/sCOD for each feed type throughout the experiment (mean ± SD, $n = 3$). No significant difference between feed types was found at day 0. SFS-P:E 15 was significantly lower than the rest of the SFS at day 7.

3.4. Nitrogen and phosphorus dissolution

The initial values for TAN released ranged between 0.01 and 0.03 g TAN/g TKN (Fig. 4). No significant differences were found in the initial (day 0) samples. The TAN/TKN ratio increased by 6.9–12.9% from day 0 to day 3 for all dietary groups, whereas afterwards the net increment was ≤7%. At day 7, the TAN/TKN yield reached values between 0.14 and 0.36 g/g, and the values for $SFS_{P:E\ 19}$, $SFS_{P:E\ 21}$, and $SFS_{P:E\ 23}$ were at this time significantly higher than $SFS_{P:E\ 15}$. The latter displayed the lowest nitrogen

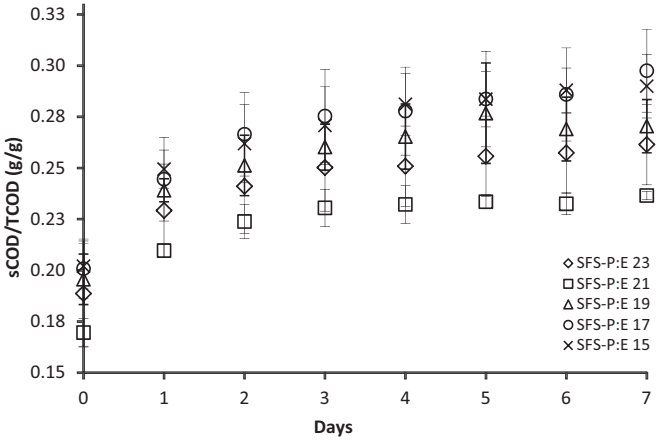


Fig. 1. Cumulative hydrolysis (degree of solubilization) throughout the experiment shown as sCOD/TCOD in SFS deriving from each feed type (mean ± SD, $n = 3$). No significant difference between feed types was found at day 0. SFS-P:E 15 and SFS-P:E 17 were significant higher compared to SFS-P:E 21 at day 7.

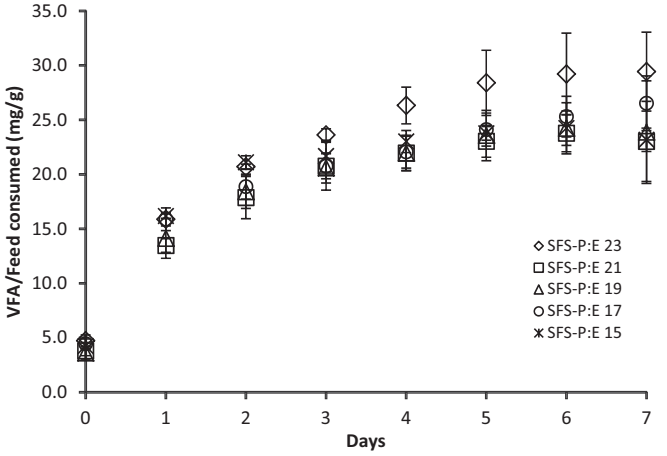


Fig. 3. Cumulative VFA mass production shown as mg VFA/g feed consumed for each feed type throughout the experiment (mean ± SD, $n = 3$). No significant differences were found at day 0 or day 7 between SFS from the different diets.

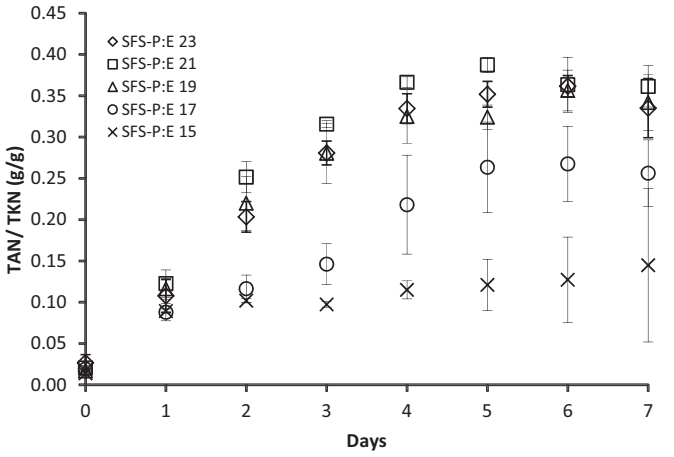


Fig. 4. Dissolution of TAN shown as g TAN/g TKN for each feed type throughout the experiment (mean ± SD, $n = 3$). No significant differences were found between feed types at day 0. SFS-P:E 19, SFS-P:E 21, and SFS-P:E 23 were significantly higher than SFS-P:E 15 at day 7.

dissolution with a net production of 0.08 g TAN during the 7 days compared to 0.24–0.27 g TAN for the other treatment groups.

The dissolution of phosphorus expressed as orthophosphate (PO_4^{3-}-P/TP) at day 0 ranged between 0.006 and 0.010 g PO_4^{3-}-P/g TP for all SFS (Fig. 5), with no significant differences between them. The PO_4^{3-}-P/TP ratio increased from day 0 to day 1 for $SFS_{P:E\ 15}$ (10.8%) and $SFS_{P:E\ 17}$ (6.5%), whereas for the rest of the dietary groups the increment was between 2.3 and 3.4% within the same period. During the rest of the evaluation the PO_4^{3-}-P/TP ratio increased by ≤3% for all dietary groups.

At day 7, the PO_4^{3-}-P/TP yield reached values between 0.03 and 0.15 g/g, and the values for $SFS_{P:E\ 15}$ (0.15 g/g) followed by $SFS_{P:E\ 17}$ (0.08 g/g) were at this time significantly higher than the other treatment groups. $SFS_{P:E\ 15}$ and $SFS_{P:E\ 17}$ reached at day 7 a PO_4^{3-}-P net production of 87.0 mg and 55.3 mg respectively, whereas the rest of the dietary groups reached a net production between 16.70 and 55.30 mg during the same time interval.

3.5. C:N ratio

The C:N ratio, an indicator of the potential capacity of the SFS produced from the different P:E diets to balance denitrification (Henze et al., 2002; Metcalf and Eddy, 2004), is expressed here as the obtained C:N ratio (equaling VFA/TKN), while the potential C:N

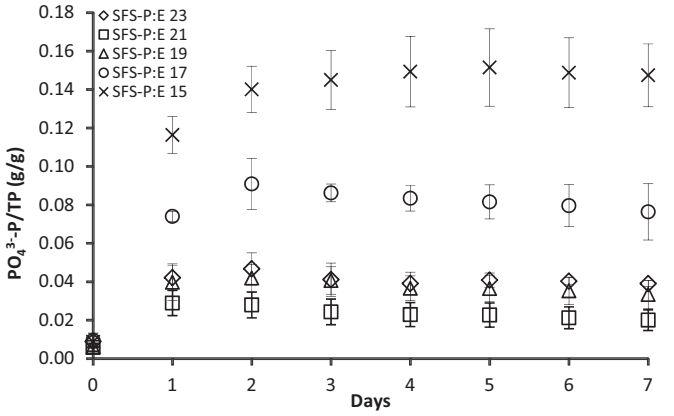


Fig. 5. Dissolution of phosphorus in relation to the TP found in SFS, expressed as PO_4^{3}-P/TP for each feed type throughout the experiment (mean ± SD, $n = 3$). No significant differences were found between feed types at day 0. SFS-P:E 15 and SFS-P:E 17 were significantly higher than the other treatment groups at day 7.

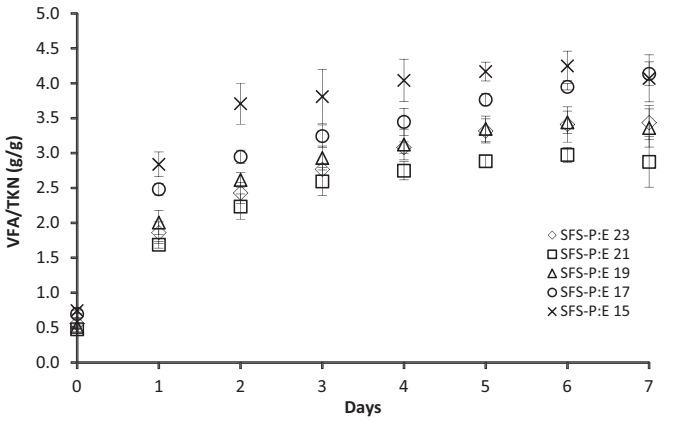


Fig. 6. Obtained C:N ratio (expressed as VFA/TKN) throughout the experiment (mean ± SD, $n = 3$). No significant differences were found between feed types at day 0. SFS-P:E 15 and SFS-P:E 17 were significantly higher than SFS-P:E 21 and SFS-P:E 17 was significantly higher than SFS-P:E 19.

ratio corresponds to TCOD/TKN. The obtained C:N ratio at day 0 ranged between 0.48 and 0.74 g VFA/g TKN (Fig. 6). No significant differences were found between feed types. At day 7, the obtained C:N ratio for all the evaluated SFS ranged between 2.87 and 4.14 g VFA/g TKN, being significantly lower for $SFS_{P:E\ 21}$ compared to $SFS_{P:E\ 17}$ and $SFS_{P:E\ 15}$. For the potential C:N ratio, values at day 7 ranged between 21.43 and 39.06 g TCOD/g TKN, with $SFS_{P:E\ 15}$ (39.06 g/g) being significantly higher than the rest of the evaluated diets.

3.6. pH

The initial pH values (day 0) for all dietary groups ranged between 7.7 and 8.1 with no significant differences between them. At day 1 all values from the different dietary groups had dropped to a range between 5.5 and 6.1 (Fig. 7), remaining constant around this level through the rest of the experiment. Significantly lower values for $SFS_{P:E\ 15}$ and $SFS_{P:E\ 17}$ was found at day 7 as compared to the rest the evaluated SFS.

3.7. Fish performance

Fish performance was generally good for all dietary treatments. The specific growth rate (SGR) increased as P:E increased, ranging between 2.10 and 2.55. Concurrently, the feed conversion

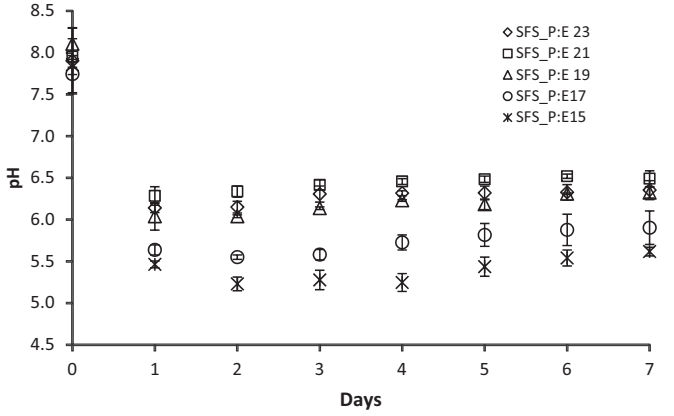


Fig. 7. pH values obtained throughout the experiment (mean ± SD, $n = 3$). No significant difference between dietary groups was found at day 0. Significant difference for SFS-P:E 15 and SFS-P:E 17 compared to the rest of the evaluated SFS was found at day 7.

ratio (FCR) decreased as P:E increased, declining from 0.82 to 0.66 (Table 2).

4. Discussion

Only few studies have so far reported on the hydrolyzation of fish sludge, and they have typically used different approaches and nomenclatures to describe the hydrolysis/fermentation process and the related dissolution of nitrogen and phosphorus (van Rijn et al., 1995; Conroy and Couturier, 2010; Suhr et al., 2013). Therefore, comparisons of results between studies can be complex. The hydrolysis process is believed to be the rate-limiting step of anaerobic digestion, which is why VFA formation in some studies has been considered almost as a result of one process (Conroy and Couturier, 2010; Suhr et al., 2013). However, in strict terms it is two, concurrent and coupled processes (solubilization and fermentation) (Eastman and Ferguson, 1981; Gerardi, 2006). In this study, the solubilization and the fermentation processes were consequently described separately, enabling a comparison of the effects of the different P:E ratios in the formulated diets on the dynamics of the two processes involved.

4.1. Hydrolysis (degree of solubilization)

The degree of solubilization obtained in the present study (sCOD/TCOD) may be re-expressed as sCOD/TVS in order to compare with previous studies, and ranged between 0.39 and 0.48 g sCOD/g TVS at day 1. In comparison, Suhr et al. (2013) found a maximum yield of 0.29–0.31 g sCOD/g TVS for fish waste collected (during 48 h) from sludge cones at a commercial, 1000 tonne/year, recirculating rainbow trout system. The maximal yield was reached at day 16 when performing hydrolysis/fermentation of the collected fish waste. Similarly, Conroy and Couturier (2010), using salmon waste that was collected after 24 h, frozen and posteriorly blended before performing hydrolysis/fermentation, reported a maximum yield of 0.4 g sCOD/g TVS at day 10. Hence, even though the values for sCOD/TVS obtained in the current study appear to agree with previous studies, the time frame for these yields to be obtained was much shorter (i.e. 1 day), presumably due to a higher fraction of easily degradable organic matter not being lost or consumed by bacteria within the system.

During the hydrolysis process, bacteria use enzymes such as proteases and amylases for degrading proteins and carbohydrates, respectively (Gerardi, 2006). In the current study hydrolytic bacteria in the SFS, likely deriving from the rainbow trout intestine, presumably hydrolyzed the particulate matter while fermentative bacteria produced acids in the form of VFA, with both processes happening simultaneously in the fermentors. The latter of the two processes may explain the observed drop in pH from 8.1-7.7 at day 0 to 6.1-5.5 at day 1 and onwards in all fermentors (Fig. 7). Furthermore and according to Hidalgo et al. (1998), the proteolytic activity in rainbow trout intestine is reduced to approximately 30% of its full activity at pH 6.0 whereas full activity is found at pH 8.5. This may explain the low degree of solubilization found in the present study from day 1 onwards (Fig. 1). Similar findings have been reported by Chen et al. (2007) and Eastman and Ferguson (1981), describing a higher solubilization of carbohydrates and proteins for wastewater treatment sludge at pH values between 7.0 and 11.0, while Cokgor et al. (2008) reported a reduction in the hydrolysis capacity in wastewater at pH values between 5.5 and 6.5.

In relation to the effect of the different dietary groups towards the degree of solubilization there appeared to be a tendency for lower P:E ratio diets (i.e. $SFS_{P:E\ 15–17}$) to produce SFS with a higher capacity for solubilization than the other dietary treatment groups. Hence, the SFS from the two former groups contained more NFE/TS (29–38%) than the high P:E diets containing 16–21% NFE/TS (Table 2). Christ et al. (2000) evaluated the hydrolysis constants at thermophilic conditions for different organic waste, and showed that carbohydrates are hydrolyzed at a faster rate ($k_{max} = 0.2\ day^{-1}$) than proteins ($k_{max} = 0.075\ day^{-1}$), which may also contribute to explaining the observed tendency in the degree of solubilization obtained in the current study.

4.2. Fermentation (VFA production)

The degree of fermentation (VFA/sCOD) reached maximum values (36–57%) at the last two days of the evaluation (i.e. day 6 and 7). These yields are lower than the values reported for wastewater treatment plants (83–99%) using primary sludge fermented for 5–7 days (Cokgor et al., 2008; Suheyl and Henze, 2008). They are also lower than described in the study by Suhr et al. (2014), reporting VFA yields generated from 5 days hydrolysis/fermentation on rainbow trout waste, reaching yields between 74 and 76% (VFA/sCOD).

Converting the obtained values from the current experiment into VFA/TVS, the VFA yield ranged between 0.17 and 0.24 g VFA/g TVS at day 6 and 7 for all SFS. These values are slightly higher than those reported by Conroy and Couturier (2010), who achieved 0.13 g VFA/g TVS at day 10, but similar to those obtained by Suhr et al. (2013), who found 0.21 and 0.15 g VFA/g TVS at day 16. Therefore, the mass of VFA produced per unit organic matter (measured as TVS) in the SFS during this experiment are in concordance with other studies, but with a higher fraction of available sCOD not being converted to VFA, amounting to 46–49% for $SFS_{P:E\ 17\text{-}19\text{-}21\text{-}23}$ and 64% in the case of $SFS_{P:E\ 15}$. The lower fermentation yield obtained might be attributed to the fact that the NMBS used in this experiment were operated under flow-through configuration. Hence, the consortia of fermentative bacteria present in the water was presumably much lower and less robust than that of RAS or wastewater treatment plants, where abundant and more diverse bacterial consortia might be found. The bacteria consortia in the current experiment presumably derived mainly from the intestine of the fish, and therefore possessed a limited fermentative activity towards a substrate that was not absorbed or digested by the fish. Supplementary, the capacity of acidogenic/fermentative bacteria to use all the available sCOD for producing VFAs might have been reduced as an accumulation of fermentation end products including primarily organic acids (i.e. VFAs) occurred, potentially creating a feedback inhibition situation as described by Gerardi (2006). Such situation is usually avoided in anaerobic digestion, where methanogenic bacteria take up the end products of the acidogenic/fermentative step (i.e. VFAs), stabilizing the pH to neutral values, and thereby allowing the continuity of the process (Gerardi, 2006).

The dietary P:E ratio had a significant effect on the degree of fermentation, seen as a lower degree of fermentation (VFA/sCOD; Fig. 2) of SFS from diet P:E 15 compared to the SFS from the other dietary groups. Interestingly, this tendency was not the same for the degree of solubilization (sCOD/TCOD; Fig. 1) where SFS from P:E 15 and P:E 17 achieved the highest dissolution yields. Hence, it appeared that hydrolysis yields were higher for SFS from lower P:E diets while the fermentation yield was significantly lower specifically for P:E 15. The inverse hydrolysis/fermentation tendency occurred in SFS from higher P:E ratio diets. From these results, two hypothesis might be put forward: (1) SFS from P:E 15 and to some extent P:E 17 possessed an intrinsic property that partly hampered fermentation as compared to SFS from higher P:E diets; and (2) the fermentation products of the lower P:E ratio diets included relatively larger shares of alcohols and other non-acid compounds which were not quantified by the analytical method applied (VFA) in this study.

Regarding the first hypothesis, diet P:E 15 and 17 contained 24.6 and 19.7% NFE, respectively compared to 8.8–13.7% in the other diets. The NFE presumably consisted primarily of starch and sugars given the dietary ingredients (Table 1). Soluble COD, the measured product of hydrolysis in this study, will include inert or non-biodegradable material. Applying especially to SFS deriving from diet P:E 15 and 17, larger shares of "hardly" biodegradable carbohydrates might have been solubilized by the hydrolytic bacteria or by stirring without being further fermented by acidogenic bacteria, as suggested by Hendriks and Zeeman (2009). Given that a 46–64% available margin for improvement on VFA production was present in this study, future studies might focus on characterizing the carbohydrates deriving from the hydrolysis process in order to elucidate what intrinsic properties may cause a reduction in the fermentation of soluble products.

Regarding the second hypothesis, the current study only quantified VFAs as potential fermentation products, expressing them as acetate units according to the test kit procedure applied. Primary fermentation products of carbohydrates (i.e. alcohols) were thus not quantified. Consequently, and for SFS from P:E 15 and 17 in particular, this implies that the obtained degree of fermentation might actually have been higher, which would had been disclosed if all fermentation products (e.g. alcohols) had been accounted for. Hence, the method applied is probably suitable for evaluating VFA production, but supplementary analysis quantifying e.g. alcohols are needed for quantification of other fermentation products, especially when carbohydrates are involved.

4.3. Nitrogen and phosphorus dissolution

The fermentation process results in a release of ammonia and phosphate to the bulk phase that may be undesirable if left untreated (Gerardi, 2006; Conroy and Couturier, 2010). However, as the hydrolysis and fermentation processes are usually performed in a fermentor, the degradation of organic matter and the release of nutrients from SFS can normally be controlled.

The dissolution yield (TAN/TKN) in the current study varied between 0.14 and 0.36 g TAN/g TKN, increasing mainly during the first 4 days of evaluation (Fig. 4) as also reported from fermentation of primary sludge in wastewater treatment plants (Cokgor et al., 2008; Suheyl and Henze, 2008; Yuan et al., 2011). For the lower P:E diets the dissolution seems to reflect the protein content in the SFS (Table 2).

The phosphorus concentrations reached in the bulk phase (17–92 mg/L) are in accordance with those reported by Courier and Couturier (2010), who found concentrations above 100 mg/L at day 1 when hydrolyzing waste from salmon. Courier and Couturier (2010) described the relation between phosphorus, dicalcium phosphate dihydrate and pH, and concluded that the dissolution of phosphorus is inversely related to pH. This is in accordance with the values obtained in the present study, where SFS deriving from diets P:E 15–17 showed a significantly higher phosphorus dissolution than the others, presumably due to the lower pH reached in the reactors. The apparent inverse tendency observed between nitrogen and phosphorus dissolution were thus likely unrelated, and were probably caused by the lower pH reached in the P:E 15–17 reactors.

4.4. C:N

The relation between the amount of carbon and nitrogen in the SFS is an important characteristic for biological nutrient removal. For single-sludge denitrification this relation defines the potential of using SFS for biologically removing nitrate from the effluents (Henze et al., 2002; Huiliñir et al., 2012). Hence, the amount of carbon available must be enough to support aerobic bacterial respiration to achieve anoxic conditions, bacterial growth (i.e. sludge production), as well as the reduction of NO_3^--N to N_2 (Henze et al., 2002). The processes are closely related to the characteristics of the SFS and the overall N-waste produced, which again is related to the diet composition and associated digestibility and utilization by the fish.

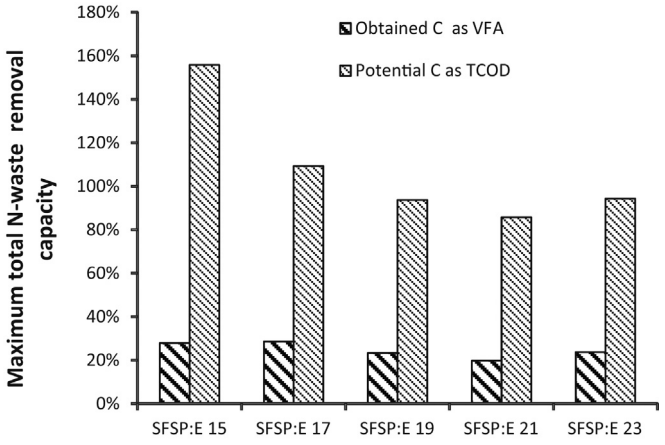


Fig. 8. Maximum capacity for nitrate removal through denitrification according to the C found in the SFS (expressed as TCOD) and the C obtained as VFA after 7 days of Hydrolysis/Fermentation process. A C:N ratio of 2.9 was used for calculating the amount of nitrate-N than could be removed using the obtained C (VFA), and a C:N ratio of 5 was used for calculating the amount of nitrate-N than theoretically could be removed using the potential C (TCOD) (Henze et al., 2002). The total N-waste produced by fish contains the N found in the SFS plus the estimated dissolved fraction of ammonia excreted by fish assuming that 80% of total N-waste is found in the dissolved/suspended fraction (Dalsgaard and Pedersen, 2011). It was assumed that 1 g NH_4^+-N is oxidized to 1 g NO_3^--N.

The amount of carbon required in the following calculations accounts for the two first processes mentioned above (i.e. denitrification and bacterial growth). As previously mentioned, the C:N ratios in this experiment were evaluated both as obtained (VFA/TKN) and as potential (TCOD/TKN) C:N ratios. In the literature, values between 2.9 and 3.2 g sCOD/g N have been stated as the optimum C:N ratio for denitrification using readily biodegradable organic matter from wastewater sludge (Henze et al., 2002). In aquaculture, values between 3.0 and 6.0 g TCOD/g NO_3^--N have been described as optimal (van Rijn et al., 2006; Suhr et al., 2013). Evaluating the available C:N ratios using the produced, readily biodegradable carbon source (VFA expressed as acetic acid units) from the hydrolysis/fermentation process (Fig. 6), all SFS reached an optimum C:N ratio of 3.4–4.1 g VFA/g TKN at days 5–6. This indicates that at least 5 days retention time should be considered when designing a hydrolysis/fermentation reactor. The potential C:N ratios ranged between 21.4 and 39.1 g TCOD/g TKN for all SFS. In particular, the SFS from diet P:E 15 displayed the highest C:N ratio, and therefore in theory had the best properties for achieving complete denitrification.

The obtained C:N ratios would in theory allow for a complete removal of the N contained in the SFS, and if considering the potential C:N ratios, there could be capacity for removing additionally 26–39 mg NO_3^--N/g feed consumed using a theoretical C:N ratio of 5. Hence, considering the potential C:N ratio achieved and assuming that 70–80% of the total N released from fish is found as suspended/dissolved matter (Dalsgaard and Pedersen, 2011), there was potentially enough carbon to remove between 86 and 156% of the N produced in the culture system (Fig. 8), although the actual removal capacity ultimately will depend on optimizing the hydrolysis/fermentation process.

Values obtained and calculations performed reflect a situation where virtually all SFS produced by the fish are collected and

utilized under anoxic conditions. In on-farm conditions, factors related to issues like solids capture efficiency, system design and dissolved oxygen (DO) must be considered for optimizing SFS collection and utilization.

5. Summary

In summary, the current study showed that by performing hydrolysis and fermentation of SFS, the availability of biodegradable carbon sources is enhanced, and a higher C:N ratio for concomitant denitrification is achieved. Lower P:E diets resulted in a higher degree of solubilization measured as sCOD, while at the same time (applying especially to diet P:E 15) a lower degree of fermentation measured as VFA was obtained, indicating differences in the fermentation process. The solubilization of phosphorus was higher for lower P:E diets presumably due to a lower pH obtained during the hydrolysis/fermentation process. The different dietary protein:energy ratios resulted in different N and TCOD masses in the SFS, affecting directly the final C:N ratio, which is essential for maximizing denitrification. In this sense, lower P:E diets showed better properties for maximized N removal since the N content in SFS was lower, enhancing the obtained as well as the potential C:N ratio. All tested diets produced settable faecal solids with the potential to produce enough carbon for removing between 26 and 39 mg NO_3^--N/g feed consumed in addition to the N already present in the SFS, corresponding to a removal of 86–156% of the total N waste produced during the fish culture process. The calculations presented here are based on maximum obtainable values, whereas the potential for collection and concomitant hydrolysis in on-farm conditions will be less and depend on e.g. the solid collection system, system configuration, and DO in the effluent to treat.

Acknowledgments

This research was funded by The Ministry of Food, Agriculture and Fisheries of Denmark and by the European Union through The European Fisheries Fund (EFF). The technical skills and invaluable assistance of Brian Møller, Ulla Sproegel and Erik Poulsen are highly appreciated.

References

Amirkolaie, K.A., 2011. Reduction in the environmental impact of waste discharged by fish farms through feed and feeding. Aquaculture 3, 19–26.
Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.
Bureau, D.P., Cho, C.Y., 1999. Phosphorus utilization by rainbow trout (*Oncorhynchus mykiss*): estimation of dissolved phosphorus waste output. Aquaculture 179, 127–140.
Chen, Y., Jiang, S., Yuan, H., Zhou, Q., Gu, G., 2007. Hydrolysis and acidification of waste sludge at different pHs. Water Res. 41, 683–689.
Christ, O., Wilderer, P.A., Angerhöfer, R., Faulstich, M., 2000. Mathematical modelling of the hydrolysis of anaerobic processes. Water Sci. Technol. 41, 61–65.
Cho, C.Y., Hynes, J.D., Wood, K.R., Yoshida, H.K., 1994. Development of high-nutrient-dense, low pollution diets and prediction of aquaculture wastes using biological approaches. Aquaculture 124, 293–305.
Cho, C.Y., Bureau, D.P., 2001. A review of diet formulation strategies and feeding systems to reduce excretory and feed wastes in aquaculture. Aquac. Res. 32, 349–360.
Cokgor, E.U., Oktay, S., Tas, D.O., Zengin, G.E., Orhon, D., 2008. Influence of pH and temperature on soluble substrate generation with primary sludge fermentation. Bioresour. Technol. 100, 380–386.
Coloso, R.M., King, K., Fletcher, J.W., Hendrix, M.A., Subramanyam, P.W., Ferraris, P., 2003. Phosphorus utilization in rainbow trout (*Oncorhynchus mykiss*) fed practical diets and its consequences on effluent phosphorus levels. Aquaculture 220, 801–820.
Conroy, J., Couturier, M., 2010. Dissolution of minerals during hydrolysis of fish waste solids. Aquaculture 298, 220–225.
Dalsgaard, J., Ekmann, K.S., Pedersen, P.B., Verlhac, V., 2009. Effect of supplemented fungal phytase on performance and phosphorus availability by phosphorus-depleted juvenile rainbow trout (*Oncorhynchus mykiss*), and on the magnitude and composition of phosphorus waste output. Aquaculture 286, 105–112.
Dalsgaard, J., Pedersen, P.B., 2011. Solid and suspended/dissolved waste (N, P, O) from rainbow trout (*Oncorhynchus mykiss*). Aquaculture 313, 92–99.
Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K., Pedersen, P.B., 2013. Farming different species in RAS in Nordic countries: current status and future perpectives. Aquac. Eng. 53, 2–13.
Danish Ministry of Environment, 2012. Bekendtgørelse om miljøgodkendelse og samtidig sagsbehandling af ferskvandsdambrug (Executive Order on the Environmental Approval and Handling of Freshwater Aquaculture Systems. Lovtidende A nr. 130.). Miljøstyrelsen, Miljøministeriet, Denmark (in Danish).
DS/EN 224, 1975. Water Analysis – Determination of Ammonia–Nitrogen (DS 224:1975). Danish Standards, 8 pp.
Eastman, J.A., Ferguson, J.F., 1981. Solubilization of particulate organic carbon during the acid phase of anaerobic digestion. J. Water Pollut. Control Fed. 53 (3), 266–352.
FAO, 2012. The State of World Fisheries and Aquactulture. Food and Agriculture Organization of the United Nations, Rome.
Gerardi, M., 2006. Wastewater Bacteria. A John Wiley & Sons, New Jersey, pp. 188.
Glavic, P., Lukman, R., 2007. Review of sustainability terms and their definitions. J. Clean. Prod. 15, 1875–1885.
Green, J.A., Hardy, R.W., 2008. The effects of dietary protein:energy ratio and amino acid pattern on nitrogen utilization and excretion of rainbow trout *Oncorhynchus mykiss* (Walbaum). J. Fish Biol. 73, 663–682.
Hamlin, H.J., Michaels, J.T., Beaulaton, C.M., Graham, W.F., Dutt, W., Steinbach, P., Losordo, T.M., Schrader, K.K., Main, K.L., 2008. Comparing denitrification rates and carbon sources in commercial scale upflow denitrification filters in aquaculture. Aquac. Eng. 38, 79–92.
Hidalgo, M.C., Urea, E., Sanz, A., 1998. Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. Aquaculture 170, 267–283.
Hendriks, A.T.W.M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. 100, 10–18.
Henze, M., Harremoës, P., Jansen, J.L.C., Arvin, E., 2002. Wastewater Treatment. Biological and Chemical Process, 3rd ed. Springer, pp. 430.
Huiliñir, C., Hernandez, S., Aspe, E., Roeckel, M., 2012. Simultaneous nitrate organic matter removal from salmon industry wastewater: the effect of C/N ratio, nitrate concentration and organic load rate on batch continuous process. J. Environ. Manag. 101, 82–91.
ISO 5983, 2005. Animal Feeding Stuffs – Determination of Nitrogen Content and Calculation of Crude Protein Content – Part 2: Block Digestion/Steam Distillation Method. International Organization for Standardization, Geneva, 14 pp.
ISO 6491, 1998. Animal Feeding Stuffs – Determination of Phosphorus Content – Spectrometric Method. International Organization for Standardization, Geneva, Switzerland, 6 pp.
ISO 6878, 2004. Water Quality – Determination of Phosphorus–Ammonium Molybdate Spectrometric Method. International Organization for Standardization, Geneva, 21 pp.
Jewell, W.J., Cummings, R.J., 1990. Expanded bed treatment of complete recycle aquaculture systems. Water Sci. Technol. 22, 443–450.
Klas, S., Mozes, N., Lahav, O., 2006. A conceptual, stoichiometry-based model for single-sludge denitrification in recirculating aquaculture systems. Aquaculture 259, 328–341.
Metcalf and Eddy, 2004. Wastewater Engineering, Treatment and Reuse, 4th ed. Mc Graw Hill.
Miron, Y., Zeeman, G., Van Lier, J.B., Lettinga, G., 1998. The role of sludge retention time in the hydrolysis and acidification of lipids, carbohydrates and proteins during digestion of primary sludge in CSTR systems. Water Res. 19, 1705–1713.
Nijhof, M., 1994. Theoretical effects of feed composition feed conversion and feed spillage on waste discharge in fish culture. J. Appl. Ichthyol. 10, 274–283.
R Core Team, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, http://www.R-project.org/
Suheyl, A., Henze, M., 2008. Biological hydrolysis and acidification of sludge under anaerobic conditions: the effect of sludge type and origin on the production and composition of volatile fatty acids. Water Res. 42, 3729–3738.
Suhr, K.I., Pedersen, P.B., Arvin, E., 2013. End-of-pipe denitrification using RAS effluent waste streams: effect of C/N-ratio and hydraulic retention time. Aquac. Eng. 53, 57–64.
Suhr, K.I., Pedersen, L.-F., Nielsen, J.L., 2014. End-of-pipe single-sludge denitrification in pilot-scale recirculating aquaculture systems. Aquac. Eng. 62, 28–35.
Timmons, M., Ebeling, J., Piedrahita, R., 2009. Acuicultura en Sistemas de Recirculación, 1st ed. Editorial CayugaAquaVentures, LLC, Ithaca, NY, pp. 959 (in Spanish).
van Rijn, J., Fonarev, N., Berkowitz, B., 1995. Anaerobic treatment of intensive fish culture effluents: digestion of fish feed and release of volatile fatty acids. Aquaculture 133, 9–20.
van Rijn, J., Tal, Y., Schreier, H., 2006. Denitrification in recirculating systems: theory and applications. Aquac. Eng. 34, 364–376.
Yuan, Q., Sparling, R., Oleszkiewicz, J.A., 2011. VFA generation from waste activated sludge: effect of temperature and mixing. Chemosphere 82, 603–607.

Paper II

Aquacultural Engineering 77 (2017) 27–32

Contents lists available at ScienceDirect

Aquacultural Engineering

journal homepage: www.elsevier.com/locate/aqua-online

Review

The composition of readily available carbon sources produced by fermentation of fish faeces is affected by dietary protein:energy ratios

Carlos O. Letelier-Gordo*, Bodil K. Larsen, Johanne Dalsgaard, Per Bovbjerg Pedersen

Technical University of Denmark, DTU Aqua, Section for Aquaculture, The North Sea Research Centre, P.O. Box 101, DK-9850 Hirtshals, Denmark

ARTICLE INFO

Article history:
Received 17 August 2016
Received in revised form 24 January 2017
Accepted 30 January 2017
Available online 4 February 2017

Keywords:
Ethanol
Fermentation
Protein:energy
Single-sludge denitrification
VFAs

ABSTRACT

Fish solid waste (faeces) produced in recirculated aquaculture systems (RAS) might be used for on-farm, single-sludge denitrification if transformed into soluble organic carbon substances. The current study investigated the effect of feeding diets with increasing protein to energy ratios (P:E_15, 17, 19, 21 and 23 g/MJ) to rainbow trout (*Oncorhynchus mykiss*) on the production of volatile fatty acids (VFAs) and ethanol during 7 days fermentation of the produced fish faeces. The total yields of VFAs and ethanol obtained (expressed as chemical oxygen demand (COD)) ranged between 0.21–0.24 gCOD/gTCOD, showing no differences between treatments. However, the type and quantities of individual VFAs and ethanol changed according to the dietary treatment. Lower P:E ratio diets resulted in higher production of butyric acid and ethanol, whereas higher P:E ratio diets resulted in an increased production of acetic and valeric acid. Changing the diet composition thus affects the composition of readily available carbon that can be derived from the faeces. This can be applied to enhance on-farm single sludge denitrification and reduce the need for adding external carbon sources such as e.g. methanol.

Contents

1. Introduction

Organic matter and nitrate are two major effluent waste products in a recirculating aquaculture system (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 costly external resources such as energy and consumables should be limited to effectively accommodate these challenges.

The "Residual Resource" approach aims at transforming waste into new resources. Single-sludge denitrification applied to RAS complies with this approach, using the organic waste produced by the fish (faecal matter) as an intrinsic electron donor for nitrate

* Corresponding author.
E-mail address: colg@aqua.dtu.dk (C.O. Letelier-Gordo).

http://dx.doi.org/10.1016/j.aquaeng.2017.01.006
0144-8609/

removal (Jewell and Cummings, 1990; van Rijn et al., 2006; Suhr et al., 2015). In this way, organic matter and nitrate are removed in the same process without a need for adding external carbon. In addition, energy for waste transportation is reduced, and most importantly the waste is treated at the end of the process chain rather than being displaced to another environment (end-of-pipe concept) (Glavic and Lukman, 2007).

Studies exploring the potential for performing single-sludge denitrification in RAS have been carried out for the last 20 years, and have included fermentation of intrinsic solid carbon sources to obtain soluble organic substances. Aboutboul et al. (1995) characterized the production of volatile fatty acids (VFAs) from direct fermentation of fish feed. In a more applied approach, Conroy and Couturier (2010) described the production of VFAs from the hydrolysis/fermentation of faecal waste, and Suhr et al. (2013) described the carbon:nitrogen (C:N) ratios required for single-sludge denitrification when using VFAs deriving from fermented fish faecal matter.

The composition of the readily available carbon (RAC) produced during the fermentation process has been shown to affect denitrification rates, sludge production, and denitrification yields (Henze, 1991). Different authors have demonstrated that denitrification rates using acetic acid or a mixture of VFAs as electron donors may double the rates as compared to methanol, which is often applied as an external carbon source in aquaculture (Fass et al., 1994; Yatong, 1996; Lee and Welander, 1996). 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 (Yatong, 1996) and bacterial yields (Constantine and Fick, 1997) have been reported when using different organic carbon sources.

The composition of RAC and the C:N ratio consequently dictate the amount of carbon required for the denitrification process, and/or the amount of bacterial biomass that will be produced.

As opposed to many other types of wastewater, faecal waste in RAS is produced in a more or less continuous and predictable manner both in terms of quantity and quality. The particulate waste is mainly composed of the undigested fractions of predefined amounts of feed fed into the system each day (i.e., input). This means that aquaculture faecal waste has good potentials 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). In continuation of this, Letelier-Gordo et al. (2015) demonstrated that fermentation of faeces from rainbow trout fed diets with different protein to energy (P:E, g/MJ) ratios produced waste with different C:N ratios. Hence, fermented faeces deriving from fish fed with the lowest P:E ratio diets had a higher potential for complying with single-sludge denitrification giving a more favorable C:N ratio.

The production and application of specific types of organic acids obtained from fish faecal waste via fermentation may consequently not only reduce the need for an external carbon source for denitrification in RAS, but may also reduce the disposal of organic waste (fish faeces). Furthermore, as the feed composition, and consequently the composition of faecal waste feeding into the waste treatment system is relatively constant, there is a high potential for predicting and estimating the yields of different types of RAC that would be produced, and subsequently be available for biological waste treatment. To explore this potential in aquaculture, the current study investigated the specific composition and temporal production dynamics of different carbon sources (VFAs and ethanol) produced during 7 days of fermentation of faeces from rainbow trout fed diets with different P:E ratios.

2. Materials and methods

2.1. Settleable faecal solids

Settleable faecal solids (SFS) from rainbow trout fed five diets with different P:E ratios (P:E_15, 17, 19, 21, 23) as described in Letelier-Gordo et al. (2015) were used to evaluate the composition and net production of RAC. The SFS were hydrolyzed and fermented for 7 days in 1L enclosed Blue Cap bottles (SCHOTT Duran, Germany). The 7 days evaluation of the hydrolysis and fermentation process was chosen with the aim of avoiding further methanogenic activity (Miron et al., 1998). The bottles were mounted with sealed screw caps including two sampling ports (cap_GL, Duran Group, Germany) to ensure anaerobic conditions also when samples were withdrawn. The resulting, anaerobic Blue Cap batch reactors were maintained at $20 \pm 2\,^{\circ}C$ and with constant stirring (200 rpm, Big Squid, IKA, Germany). Samples of 50 mL were obtained at day 0 for SFS characterization (TCOD, total Kjeldahl nitrogen (TKN), lipids, and ash), and daily samples of 30 mL were taken for analysis of the RAC produced (VFAs and ethanol (Eth)). Measurements of pH and temperature were made on each daily sample using a Hatch HQ40d (Hach Lange GMbH, Dusseldorf, Germany).The characteristics of the different SFS were normalized to the mass of TCOD measured in each reactor at the beginning of the fermentation process (i.e., day 0; Table 1).

2.2. Analytical methods

Daily samples were centrifuged at 4500 rpm for 15 min at $0\,^{\circ}C$ immediately after they were obtained, and filtered through 0.2 μm syringe filters (Filtropour S, SARSTEDT, Germany). Filtered samples for VFA and ethanol analysis were subsequently preserved by adding 1% v/v sulfuric acid (4 Mol/L H_2SO_4, Merck Millipore, Germany), and maintained at $4\,^{\circ}C$ until further analysis. The fatty acid composition of the VFAs was determined using a Perkin Elmer® Flexar® FX-15 UHPLC system fitted with a Flexar UV/Vis detector (PerkinElmer, USA). Separation was achieved using an ion exclusion column (Aminex HPX-87H 9 μm, 300 mm × 7.8 mm, Biorad, USA) installed subsequent to a guard column (Micro-Guard Cation H cartridge, Biorad, USA). The mobile phase (0.005 M H_2SO_4) was run at a flow rate of 0.7 mL/min for a total of 60 min at $55\,^{\circ}C$. The detector was operated at 210 nm, and VFAs were quantified using individual standard curves for each VFA. Each curve included 7 standards (plus 0; Sigma-Aldrich, Germany) ranging from 0.031 to 2 g/L for acetic acid (HAc), 0.0156–1 g/L for propionic acid (HPro), and from 0.0078 to 0.5 g/L for formic acid (HFo), butyric acid (HBu), and valeric acid (HVa). Ethanol (Eth) was analyzed using a "Megazyme Ethanol Assay Procedure" (K-ETOH 01/14, Megazyme international Ireland Ltd, Ireland) and measured at 340 nm using a HACH Lange spectrophotometer (DR2800, HACH Lange, Germany).

Determination of TCOD was performed using digestion vials (LCK 914, Hach Lange, Germany), TKN was determined by digesting and distilling the samples according to ISO 5983-2 (2005), and protein was calculated as crude protein = 6.25*Kjeldahl N. Lipids were analyzed according to Bligh and Dyer (1959); totals solids (TS) and ash as described in Apha (1995); and nitrogen free extract (NFE) was calculated as: NFE = TS − protein − lipid − ash.

2.3. Data treatment and statistical analysis

The total yield of RAC for each dietary treatment group was calculated as the sum of the individual VFAs and ethanol expressed on a COD basis (i.e., the stoichiometric value of COD per unit mass

Table 1
Characteristics of settleable faecal solids (SFS) produced by rainbow trout fed diets with different protein:energy ratios (P:E). Data are based on 4 × 24 h sampling and pooling of SFS as described in Letelier-Gordo et al. (2015), and values are expressed as masses produced per mass of measured TCOD (mg/g; mean ± SD, n = 3).[1,4]

Diet	P:E_15	P:E_17	P:E_19	P:E_21	PE:_23
Dry matter	$840^{a} \pm 30$	$980^{a} \pm 30$	$1030^{b} \pm 90$	$1080^{b} \pm 60$	$990^{a} \pm 50$
Protein[2]	$162.3^{a} \pm 14.7$	$233.6^{b} \pm 18.0$	$272.9^{bc} \pm 26.9$	$296.8^{c} \pm 22.3$	$266.1^{bc} \pm 18.5$
Lipid	$131.0^{a} \pm 6.7$	$119.1^{a} \pm 15.6$	$130.9^{a} \pm 11.0$	$138.2^{a} \pm 21.8$	$139.3^{a} \pm 15.9$
NFE[3]	$317.1^{a} \pm 14.9$	$285.1^{a} \pm 12.0$	$218.0^{b} \pm 22.7$	$171.3^{c} \pm 12.9$	$194.5^{bc} \pm 13.0$
Ash	$230.6^{a} \pm 11.9$	$338.3^{b} \pm 16.8$	$407.2^{bc} \pm 51.6$	$472.6^{c} \pm 48.5$	$385.8^{bc} \pm 21.4$
TCOD (g/g)[4]	21.8 ± 2.7	17.2 ± 1.7	17.0 ± 3.7	17.7 ± 2.0	20.9 ± 1.4

[1] Values within rows not sharing a common superscript were significantly different (Tukey-Kramer, $P < 0.05$).
[2] Protein was derived as total Kjeldahl nitrogen (TKN) multiplied by 6.25.
[3] Nitrogen free extract (NFE) was calculated as: NFE = total solids – protein – lipid – ash.
[4] Values correspond to g total chemical oxygen demand (TCOD)/g wet weight.

(g/g) of the different organic compounds) following Henze et al. (2008) (formic acid_COD: 0.35; acetic acid_COD: 1.07; propionic acid_COD: 1.51; butyric acid_COD: 1.82, valeric acid_COD: 2.04 and ethanol_COD: 2.09) and normalized to the masses of TCOD measured in the SFS (Table 1).

To test for significant differences between dietary treatments, comparisons between SFS characterized at day 0, the yields of RAC (VFAs and ethanol) obtained at day 7, and the pH values measured at day 0 and day 7, respectively, were performed using one-way ANOVA analysis followed by Tukey-Kramer multi comparison of means test with 95% family-wise confidence level. The statistical analyses were carried out using the R software version 3 (R Core Team, 2013).

3. Results

3.1. Effect of dietary P:E ratios on the production of individual RACs

The distribution and quantities of VFAs and ethanol changed throughout the various days of fermentation as well as in relation to the dietary treatment/diet composition (Fig. 1). In addition to ethanol (Eth), the following VFAs were identified in all groups: butyric (HBu), acetic (HAc), propionic (HPr), valeric (HVa), and formic acid (HFo).

Acetic acid was the main VFA produced after 1 day of fermentation in all treatments, ranging between 43 and 62% of the total RACS identified (Fig. 1). The net production continued to increase in all groups except for treatment group P:E_15, where the production stagnated after 2 days.

A high production of valeric acid was observed especially in treatment groups P:E_21 after 1 day of fermentation (23% of the total RACS produced in this group compared to 3–6% in the other groups), accompanied by a comparatively high production in treatment groups P:E_19 and 23 from day 2 and onwards (Fig. 1).

Propionic acid was produced in moderate amounts in all groups throughout the measuring period (Fig. 1), while formic acid was the least produced RAC, being produced mainly during the first day of fermentation (Fig. 1).

Ethanol constituted 9–20% of the total RAC produced after day 1 in the different treatment groups, and there continued to be a net production until day 2–4, at which time it was replaced by a net consumption except for treatment group P:E_15 (Fig. 1).

3.2. pH

In the currently study, pH ranged between 7.7 and 8.1 at day 0 with no differences between dietary treatment groups (Table 2). After day 1, pH had dropped to 5.5–6.3 in all groups and remained in this interval until the end of the trial (day 7), at which time it was significantly lower in dietary treatment groups P:E_15 and 17 than in the other 3 groups.

3.3. Total yields after 7 days fermentation

Fig. 2 summarize the net distribution of individual RACs produced, and the yields obtained (expressed as gRAC_COD/gTCOD) at the end of the 7 days fermentation process. Overall yields ranged between 0.21 and 0.24 g RAC_COD/gTCOD with no statistical difference between feed types. Acetic acid was the main VFA produced in dietary treatment groups P:E_21-23, accounting for approximately 40% of the total RAC. In comparison, butyric acid was the main VFA produced in P:E_15 and 17 (60 and 37%, respectively). Valeric, propionic and formic acid were recovered in all groups in lesser amounts (4–23%, 10–22%, and ≤3%, respectively), while ethanol was still recovered only in treatment groups P:E_15-17 after 7 days (13 and 3% of total RAC, respectively).

To normalize and compare each dietary treatment Table 3 estimates the specific masses of RAC (kg per ton fish produced) that may be obtained for each dietary treatment group, derived by extrapolating the data from day 7 (Fig. 2) and considering the daily amount of TCOD that was generated in the trial.

According to this, fermentation of faeces from diet P:E_15 resulted in the production of approximately 1.4 times more RAC than the other diets due to the higher mass of TCOD produced per mass of fish growth. However, when yields of RAC are normalized to TCOD obtained, all dietary treatments reached similar values between 21 and 23%.

4. Discussion

4.1. Effect of dietary P:E ratios on the production of individual RACs

To our knowledge, this is the first study to relate fish feed composition to the production of specific VFAs and ethanol in appertaining fermented fish faeces. In one study, van Rijn et al. (1995) characterized the fermentation products from fish feed, finding acetate, propionate, and butyrate (43, 36, and 21%, respectively, of total VFAs) after 24 h fermentation (201.73 mg COD/L/day). A high production of acetic acid during the first 24 h was also observed in the current study (43–62% of the total RAC identified), examining fermentation products of SFS in contrast to feed (Fig. 1). Furthermore, the net production of acetic acid continued to increase in all groups (except for treatment group P:E_15) during the first 3–4 days of fermentation and then levelled out. According to the "Anaerobic Digestion Model No1" (ADM1) developed by Batstone et al. (2002), acetic acid can be produced through several pathways (Batstone et al., 2002; Metcalf, 2004; Henze et al., 2008), explaining the high production in all treatment groups independently of the nutrient composition of the substrate (SFS).

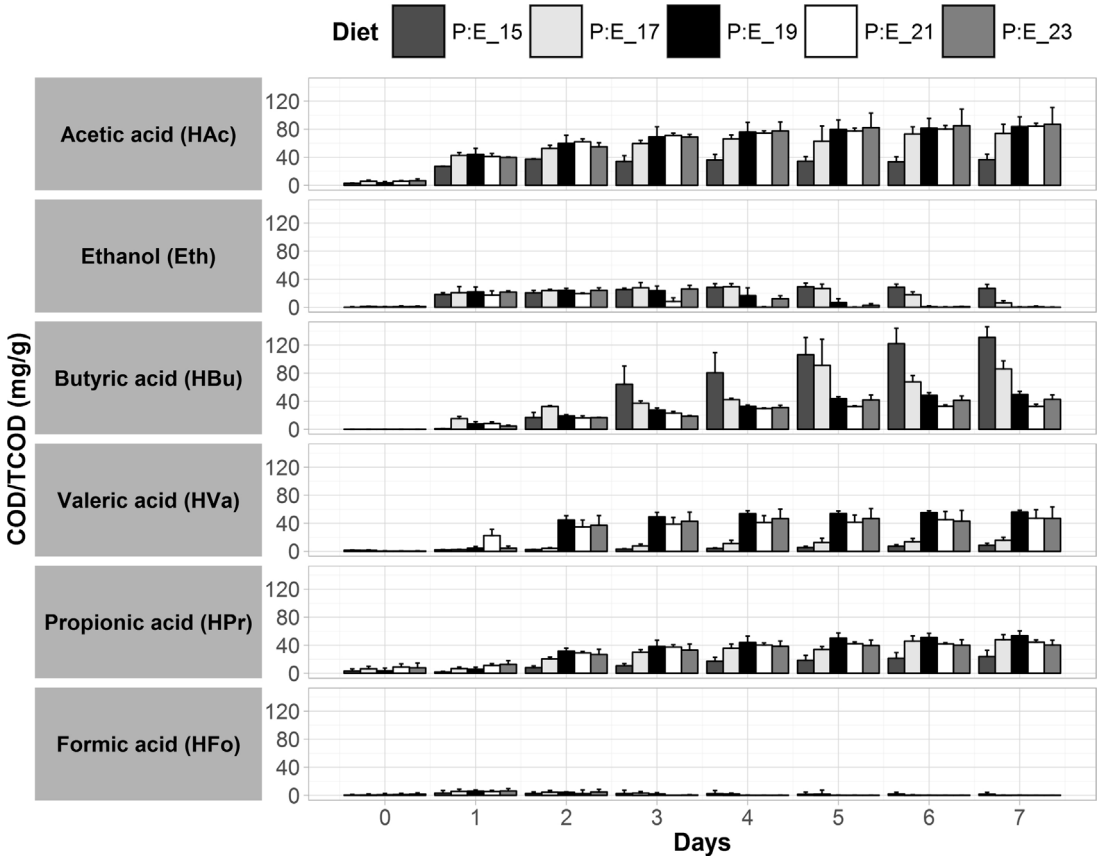


Fig. 1. Temporal pattern in the net production of acetic acid (HAc); ethanol (Eth); butyric acid (HBu); valeric acid (HVa); propionic acid (HPr) and formic acid (HFo) during 7 days of fermentation of the settleable faecal solids (SFS) deriving from different dietary treatment groups. Data are presented as COD values and normalized to TCOD measured in the samples (mean ± SD, n = 3).

Table 2
pH values measured daily from day 0–7 in the fermentation batch reactors with settleable faecal solids deriving from five dietary treatments (P:E_15–23) during the experimental period (mean ± SD, n = 3).[1]

Day/diet	P:E_15	P:E_17	P:E_19	P:E_21	P:E_23
0	7.9 ± 0.0	7.7 ± 0.2	8.1 ± 0.2	8.0 ± 0.2	7.9 ± 0.4
1	5.5^{a} ± 0.0	5.6^{a} ± 0.1	6.0^{b} ± 0.2	6.3^{b} ± 0.1	6.1^{b} ± 0.1
2	5.2^{a} ± 0.1	5.6^{a} ± 0.0	6.0^{b} ± 0.0	6.3^{b} ± 0.1	6.2^{b} ± 0.1
3	5.3^{a} ± 0.1	5.6^{a} ± 0.1	6.1^{b} ± 0.0	6.4^{b} ± 0.1	6.3^{b} ± 0.1
4	5.2^{a} ± 0.1	5.7^{a} ± 0.1	6.2^{b} ± 0.0	6.5^{b} ± 0.0	6.3^{b} ± 0.0
5	5.4^{a} ± 0.1	5.8^{a} ± 0.1	6.2^{b} ± 0.1	6.5^{b} ± 0.0	6.3^{b} ± 0.1
6	5.5^{a} ± 0.1	5.9^{a} ± 0.2	6.3^{b} ± 0.0	6.5^{b} ± 0.0	6.3^{b} ± 0.1
7	5.6^{a} ± 0.1	5.9^{a} ± 0.2	6.3^{b} ± 0.1	6.5^{b} ± 0.1	6.4^{b} ± 0.1

[1] Values within a row not sharing a common superscript letter were significantly different (Tukey-Kramer, P < 0.05).

Table 3
Masses of readily available carbon (RAC) products[c] obtained after 7 days of fermentation of the settleable faecal solids.

	FCR[a]	TCOD/Fish produced[b] (ton/ton)	kg RAC[c]/ton fish produced							
			HAc	Eth	HBu	HVa	HPr	HFo	Total	RAC/TCOD (g/g)
P:E_15	0.82	0.18	6.7	5.0	24.3	1.5	4.2	0.3	42.1	0.23
P:E_17	0.75	0.13	9.6	0.8	11.1	2.0	6.3	0.0	29.9	0.23
P:E_19	0.74	0.12	10.0	0.0	6.2	6.9	6.4	0.0	29.7	0.24
P:E_21	0.67	0.11	9.6	0.1	3.7	5.2	5.1	0.0	23.8	0.21
P:E_23	0.66	0.13	11.6	0.0	5.6	6.4	5.3	0.0	29.1	0.22

[a] Feed conversion ratios (feed consumed/biomass gain) obtained during the experiment (from Letelier-Gordo et al., 2015).
[b] Total COD (TCOD)/fish produced calculated using the yields of TCOD/feed consumed (from Letelier-Gordo et al., 2015) multiplied by the associated FCR obtained.
[c] See Fig. 1 for abbreviations.

Generally, the net production of the different VFAs changed after 2–3 days of fermentation when comparing the different treatment groups, indicating that the bacteria shifted to different pathways according to the substrate available. Hence, butyric acid was produced in particularly high amounts in the lowest P:E ratio treatment group (P:E_15), while a continuous high net production of acetic and valeric acid was observed in the highest P:E treatment groups (P:E_19, 21 and 23) (Fig. 1). According to the ADM1 model,

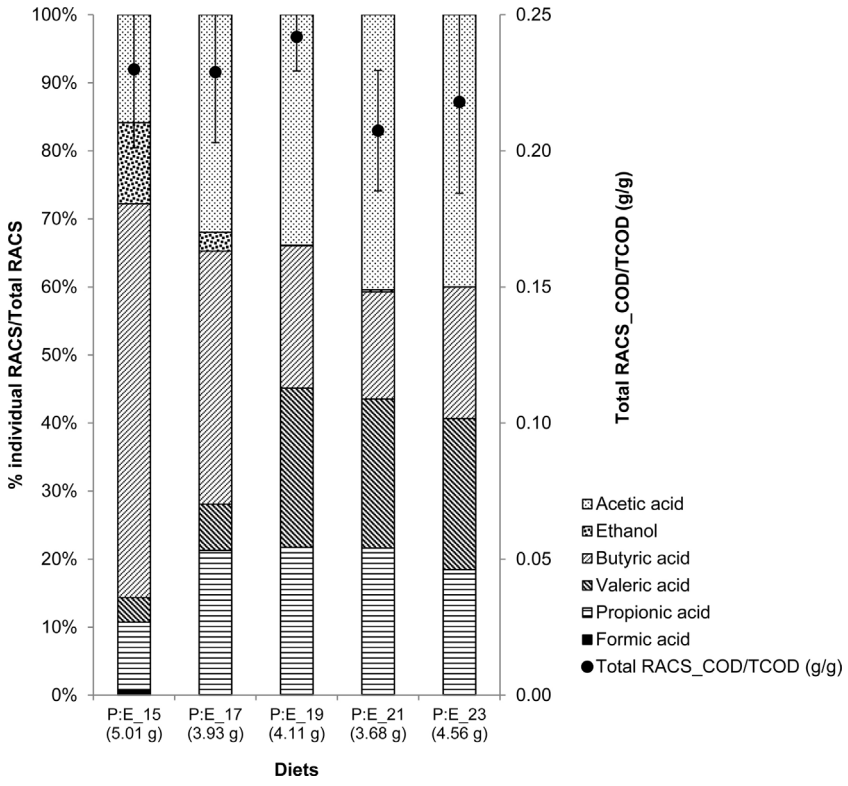


Fig. 2. Total quantities and composition of readily available carbon (RAC) products measured after 7 days of fermentation of the settleable faecal solids (SFS) deriving from different dietary treatment groups. The proportion of individual RAC products is shown as% on the left axis (mean, n = 3), while the total yields of RAC (g RAC/g TCOD) are shown on the right axis (mean ± SD, n = 3). Obtained RAC (expressed as g COD/g wet sample) are displayed in brackets on the X axis. The yields of formic, acetic and butyric acid as well as ethanol in dietary treatment P:E_15 differs significantly from the other dietary treatments. Furthermore, the yields of valeric acid in dietary treatment P:E_19, 21 and 23 differs significantly from P:E_15 and 17.

butyric and valeric acid can be produced from acidogenesis of sugars or amino acids. Acidogenesis of amino acids was thus probably the predominant bacterial pathway leading to the production of valeric acid, given that it was primarily recovered in SFS from fish fed diets with the lowest content of carbohydrates and the highest content of proteins (i.e., diet P:E_19, 21 and 23). In contrast, acidogenesis of sugars was probably the main bacterial pathway leading to the production of butyric acid, as the production of this acid was highest in the treatment groups deriving from fish fed diets rich in NFE (i.e., carbohydrates; P:E_15 and 17; Table 1).

As for the VFAs, the different content of NFE in the five treatment groups presumably explains the observed differences in the production of ethanol. Hence, production of ethanol continued to be high only in the lower P:E ratio treatment groups (P:E_15 and 17) where the NFE content (NFE/TCOD) in the SFS was highest (Table 1).

During the fermentation process the main aim is to achieve a balance between maximizing the production of RACs (VFAs and ethanol) yet avoiding methanogenesis. Previous studies have reported that a maximum yield of VFA in fish faecal waste may be achieved after 5 days of hydrolysis/fermentation (Conroy and Couturier, 2010; Suhr et al., 2014). Consistent with this, the total yield of RAC in the current study did not differ significantly from day 5 up to day 7 in any of the treatment groups and only a marginal increase was observed after day 4.

Accumulation of intermediate RAC products (specifically butyric, valeric and propionic acids) were observed in the current study, sustaining that an incomplete anaerobic process was achieved, corroborated by the daily dynamics of the individual VFAs and ethanol. Hence, the net production of acetic acid did not increase in the treatment groups after day 3 whereas the net production of formic acid decreased (i.e., it was consumed) in all groups after day 1. Acetic and formic acid are reduced end products in an anaerobic digestion process, and the consumption of formic acid, the stabilization of acetic acid net production, and the accumulation of intermediate organic acids are all major indicators of an incomplete anaerobic process.

A complex food web is involved in a complete anaerobic digestion process, including a strict relationship between different bacteria. Since hydrogen consuming acetogenic bacteria and methanogenic populations are most likely not well established in the reactors, the interspecies hydrogen transfer process is not fulfilled (Metcalf, 2004; Henze et al., 2008). In the currently study, pH ranged between 7.7 and 8.1 at day 0 with no differences between dietary treatment groups (Table 2).

The initial drop in pH was likely due to hydrogen produced by acidogens and other anaerobic bacteria and subsequently accumulating in the reactors (Table 2). A low pH affects the free energy change (positive ΔG°) and prevents the bacteria from further converting propionate and butyrate into acetate (McCarty and Smith, 1986; Metcalf, 2004). This probably explains the stagnant net production of acetic acid after the first three days and the simultaneous accumulation of intermediate organic acids including propionate and butyrate. The tendency was most evident for P:E_15 and P:E_17 having the lowest pH values.

4.2. Effects of the different dietary treatments on the denitrification process

Production and accumulation of RAC obtained via fermentation of SFS will affect the performance of a concomitant denitrification process due to different C:N ratios, denitrification rates, and sludge production. It is usually stated that a C:N ratio of 4–6 (TCOD/NO_3) is required for optimal denitrification rates when using aquaculture SFS in practice (Klas et al., 2006; Suhr et al., 2013). Yatong

(1996), however, reported lower C:N ratios when using either a mixture of VFAs (C:N value of 2.37) or individual VFAs such as acetic acid, butyric acid, ethanol, and valeric acid (C:N values of 2.05, 1.79, 1.72 and 1.91, respectively). Using different RAC sources may thus reduce the C:N ratio required for optimal denitrification.

Applying the C:N ratios reported by Yatong (1996) to the individual RAC yields obtained in the current study, it can be estimated that the different treatment groups would be able to remove 0.55–0.57 g N/g RAC produced. Even though the amounts of N that can be removed appear to be quite similar between treatments, the capacity to reduce discharged N will be quite different. Higher P:E ratio diets thus result in a higher production of faecal N waste than lower P:E diets (Letelier-Gordo et al., 2015). Diet P:E_15, for example, will result in the production of enough RAC to theoretically remove 5.1 times the faecal N waste produced in this treatment group, whereas P:E_23 will produce enough RAC to reduce 2.8 times the faecal N waste produced in that group.

In addition to C:N ratios, previous studies have shown that the type of carbon can affect the denitrification rate. Acetic acid by itself or a mixture of VFAs (including acetic, valeric, propionic and butyric acids) may for example increase denitrification rates compared to methanol, often used as an external carbon source in aquaculture (Akunna et al., 1993; Lee and Welander, 1996). It may therefore be anticipated that the different yields of RAC produced could have an impact on the denitrification rates that can be achieved, and that diets resulting in a higher production of acetic acid might sustain comparatively higher denitrification rates. In contrast, diets resulting in a high production of propionic acid (e.g. P:E_19) might reduce the denitrification rates given that propionate is the last metabolized VFA compared to acetic, valeric and butyric acids (Fass et al., 1994; Elefsiniotis and Wareham, 2007).

Using ethanol, methanol or carbohydrates (such as crude syrup, hydrolyzed starch and molasses) as substrate for denitrification increases sludge production compared to using acetic acid (Constantine and Fick, 1997; Hamlin et al., 2008). Sludge is in most cases unwanted as it needs to be disposed. Treatment group P:E_15 and 17 resulted in higher amounts of ethanol, which could favor a higher sludge production and lower denitrification rates given that bacteria need to oxidize ethanol to acetate before it is available for denitrification (Lee and Welander, 1996). In that respect diets rich in carbohydrate may not be the most efficient source for single-sludge denitrification.

5. Conclusion

The composition of fish feed qualitatively and quantitatively affected the RAC that were obtained following fermentation of the faecal waste produced. Lower levels of protein:energy in the diets (P:E_15 and 17) resulted in a higher production of butyric acid and ethanol, while higher levels of protein:energy (P:E_21 and 23) resulted in more acetic and valeric acid being produced. However, no differences in total RAC yields were found between treatments (0.21–0.24 gRAC/gTCOD). Further studies should focus on ways to optimize RAC/TCOD yields from aquaculture waste, and evaluate the effects that different RAC types and quantities have on denitrification rates, C:N ratios, and sludge production. By that, a (complete or partial) shift from applying external carbon sources to utilizing internal carbon sources (faecal waste) for denitrification in intensive fish farms could eventually be achieved.

Acknowledgments

This research was funded by The Ministry of Food, Agriculture and Fisheries of Denmark and by the European Union through The European Fisheries Fund (EFF). The technical skills and invaluable assistance of Sarah Møller Nielsen, Brian Møller and Ulla Sproegel are highly appreciated.

References

Aboutboul, Y., Arviv, R., van Rijn, J., 1995. Anaerobic treatment of intensive fish culture effluents: volatile fatty acid mediated denitrification. Aquaculture 133 (1), 21–32.
Akunna, J.C., Bizeau, C., Moletta, R., 1993. Nitrate and nitrite reductions with anaerobic sludge using various carbon sources: glucose, glycerol, acetic acid, lactic acid and methanol. Water Res. 27 (8), 1303–1312.
Apha, 1995. Stardard Methods for the Examination of Water and Wastewater, 19th ed. American Public Health Association, Washington, DC.
Batstone, D.J., Keller, J., Angelidaki, I., Kalyuzhnyi, S.V., Pavlostathis, S.G., Rozzi, A., Sanders, W.T.M., Siegrist, H., Vvili, V.A., 2002. The IWA anaerobic digestion model No 1 (ADM1). Water Sci. Technol. 45 (10), 65–73.
Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37 (8), 911–917.
Conroy, J., Couturier, M., 2010. Dissolution of minerals during hydrolysis of fish waste solids. Aquaculture 298 (3), 220–225.
Constantine, H., Fick, M., 1997. Influence of C-sources on the denitrification rate of high-nitrate concentrated industrial wastewater. Water Res. 31 (3), 583–589.
Dalsgaard, J., Pedersen, P.B., 2011. Solid and suspended/dissolved waste (N, P, O) from rainbow trout (*Oncorynchus mykiss*). Aquaculture 313 (1), 92–99.
Elefsiniotis, P., Wareham, D.G., 2007. Utilization patterns of volatile fatty acids in the denitrification reaction. Enzyme Microb. Technol. 41 (1), 92–97.
Fass, S., Ganaye, V., Urbain, V., Manem, J., Block, J.C., 1994. Volatile fatty acids as organic carbon sources in denitrification. Environ. Technol. 15 (5), 459–467.
Glavic, P., Lukman, R., 2007. Review of sustainability terms and their definitions. J. Clean. Prod. 15 (18), 1875–1885.
Hamlin, H.J., Michaels, J.T., Beaulaton, C.M., Graham, W.F., Dutt, W., Steinbach, P., Losordo, T.M., Schrader, K.K., Main, K.L., 2008. Comparing denitrification rates and carbon sources in commercial scale upflow denitrification filters in aquaculture. Aquacult. Eng. 38 (2), 79–92.
Henze, M., van Loosdrecht, M., Ekama, G.A., Brjanovic, D., 2008. Biological Wastewater Treatment: Principles, Modelling and Design, 2008. IWA Publishing, 511 pp.
Henze, M., 1991. Capabilities of biological nitrogen removal processes from wastewater. Water Sci. Technol. 23 (4–6), 669–679.
ISO 5983, 2005. Animal Feeding Stuffs—Determination of Nitrogen Content and Calculation of Crude Protein Content—Part 2: Block Digestion/Steam Distillation Method. International Organization for Standardization, Geneva, Switzerland, 14 pp.
Jewell, W.J., Cummings, R.J., 1990. Expanded bed treatment of complete recycle aquaculture systems. Water Sci. Technol. 22 (1–2), 443–450.
Klas, S., Mozes, N., Lahav, O., 2006. A conceptual, stoichiometry-based model for single-sludge denitrification in recirculating aquaculture systems. Aquaculture 259 (1), 328–341.
Lee, N., Welander, T., 1996. The effect of different carbon sources on respiratory denitrification in biological waste water. J. Ferment. Bioeng. 83 (3), 277–285.
Letelier-Gordo, C.O., Dalsgaard, J., Suhr, K., Ekmann, K.S., Pedersen, P.B., 2015. Reducing the dietary protein:energy (P:E) ratio changes solubilization and fermentation of rainbow trout (*Oncorhynchus mykiss*) faeces. Aquacult. Eng. 66, 22–29.
McCarty, P.L., Smith, D.P., 1986. Anaerobic wastewater treatment. Environ. Sci. Technol. 20 (12), 1200–1206.
Metcalf, E., 2004. Wastewater Engineering, Treatment and Reuse, 4th ed. McGraw Hill, 1819 pp.
Miron, Y., Zeeman, G., Van Lier, J.B., Lettinga, G., 1998. The role of sludge retention time in the hydrolysis and acidification of lipids, carbohydrates and proteins during digestion of primary sludge in CSTR systems. Water Res. 19, 1705–1713.
R Core Team, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0 http://www.R-project.org/.
Suhr, K.I., Pedersen, P.B., Arvin, E., 2013. End-of-pipe denitrification using RAS effluent waste streams: effect of C/N-ratio and hydraulic retention time. Aquacult. Eng. 53, 57–64.
Suhr, K., Pedersen, L.F., Nielsen, J.L., 2014. End-of-pipe single-sludge denitrification in pilot-scale recirculating aquaculture systems. Aquacult. Eng. 62, 28–35.
Suhr, K., Letelier-Gordo, C.O., Lund, I., 2015. Anaerobic digestion of solid waste in RAS: effect of reactor type on the biochemical acidogenic potential (BAP) and assessment of the biochemical methane potential (BMP) by a batch essay. Aquacult. Eng. 65, 65–71.
Yatong, X., 1996. Volatile fatty acids carbon source for biological denitrification. J. Environ. Sci. 8, 257–268.
van Rijn, J., Fonarev, N., Berkowitz, B., 1995. Anaerobic treatment of intensive fish culture effluents: digestion of fish feed and release of volatile fatty acids. Aquaculture 133 (1), 9–20.
van Rijn, J., Tal, Y., Schreier, H.J., 2006. Denitrification in recirculating systems: theory and application. Aquacult. Eng. 34 (3), 364–376.

Paper III (manuscript)

Soybean meal in rainbow trout (*Oncorhynchus mykiss*) feed: Effects on nutrient utilization, waste production, and waste treatment potential

Abstract

The following study examined the interrelationship between nutrient utilization and waste production (including masses and form) by juvenile rainbow trout (*Oncorhynchus mykiss*) fed increasing levels of soybean meal (SBM) as replacement for fish meal (FM). In addition, as settled fish feces may potentially be used as an electron donor for denitrification (i.e., N removal), the study evaluated the waste treatment potential of the organic solid waste deriving from increasingly plant-based diets. Each of five diets with increasing concentrations (10, 20, 30, 40 and 50%) of solvent extracted, toasted, high-protein (48%) SBM and a digestible protein:digestible energy (DP:DE) ratio of 20 g/MJ were fed to triplicate tanks. In addition, a pure fish meal diet (FM) was fed to triplicate tanks as a reference. The tanks were mounted in a nutrient mass-balance setup, enabling full control of the fed nutrients and the solid, dissolved and particulate waste nitrogen (N), phosphorous (P), and organic matter produced. Including more than 30% SBM in the diet depressed the growth performance of the fish and was accompanied by significant increases in the specific production of total and especially particulate organic matter waste measured as the chemical oxygen demand (COD) and 5-day biological oxygen demand (BOD_5). In contrast, increasing the inclusion level of SBM forced the fish to maximize their uptake of phosphorous (P) presumably deriving from the dietary FM fraction, resulting in significant decreases in all waste P fractions. Dietary SBM as compared to FM significantly affected the composition and masses of volatile fatty acids (VFA) and ethanol deriving from the organic matter waste following 7 days of hydrolysis / fermentation. Whereas the degree of fermentation (VFA_sCOD/sCOD) found in the dietary FM was significantly higher than obtained in dietary SBM, the increased specific production of ethanol in dietary SBM enhanced the estimated denitrification potential as a lower C:N is required for the process.

In conclusion the study showed that increasing the concentration of dietary SBM not only affected the performance of the fish but also led to a deterioration of the water quality *qua* the especially particle waste produced. In contrast, dietary SBM improved the denitrification potential of the solid waste compared to that deriving from a FM-based diet.

1.0 Introduction

The physical form (solid and dissolved) and nutritional composition of aquaculture waste is inextricably linked with the ingredients and nutritional composition of the feed (Bureau and Hua, 2010; Dalsgaard and Pedersen, 2011). This is increasingly realized by the fish feed industry and has, combined with the increasing shortage of fish meal, spurred the industry to continuously improve the recipes. Dietary feed efficiencies have consequently been significantly improved during the last three decades by optimizing the digestible protein (DP):digestible energy (DE) content of the feed, and the masses of solid and dissolved waste have as a result been significantly reduced (Bureau and Hua, 2010).

A recent study showed that changing the DP:DE content of a fish meal based diet besides affecting the feed utilization and masses of waste produced, also affected the masses of volatile fatty acids (VFA) that may be obtained from the solid waste (Letelier-Gordo et al., 2015). Volatile fatty acids can be applied as electron donors for nitrogen (N) removal via end-of-pipe, single-sludge denitrification (Klas et al., 2006), and fish feed can thus in principle be manipulated not only to improve fish performance and reduce waste production, but also to improve the biological treatment potential of the solid waste.

Numerous plant-based ingredients have been investigated as potential, sustainable, partial or fully replacements for fish meal (Hardy, 1996, 2010; Brinker and Reiter, 2011; Collins et al., 2013a). Soybean meal (SBM) is one of the best studied and often applied fish meal (FM) replacements in rainbow trout (*Onchorynchus mykiss*) diets given a high availability (USSEC, 2008; FAO, 2016) and competitive price, combined with a high protein content and reasonable balanced amino acid profile (reviewed by Gatlin et al. 2007). The apparent protein digestibility of SBM may be as good as that of FM, whereas the apparent digestibility of fat is typically reduced (Oliva-Teles et al. 1994; Storebakken et al. 2000; Ogunkoya et al., 2005; Romarheim et al. 2006). Comparing several studies using a meta-analysis approach and nutritional model simulations, Hua and Bureau (2012) found that including SBM up to a certain threshold (37 ± 6%) does not affect the growth of rainbow trout, while growth is depressed in a linear manner above the threshold inclusion level. In comparison, Collins et al. (2012) found negative quadratic relationships between the specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER), respectively of rainbow trout fed SBM up to an inclusion level of 300 g/kg.

The many studies on SBM have primarily focused on the effects on rainbow trout performance and nutrient utilization, while few studies have addressed the effects on waste production and form. Yang et al. (2011) found that replacing up to 80% of FM with phytase pre-treated SBM increased the excretion of ammonia nitrogen while it reduced the excretion of total phosphorous by fingerling rainbow trout. Ogunkoya et al. (2006) showed that increasing the dietary inclusion level of SBM from 0 to 200 g/kg increased the (estimated) excretion of dissolved nitrogen and phosphorous, reduced the (estimated) excretion of faecal nitrogen waste, and reduced the sinking speed of rainbow trout feces. The latter is particularly important with respect to producing fish in recirculating aquaculture system (RAS) where rapid and efficient removal of waste (both solid and dissolved) is essential for sustaining a high water quality (Brinker and Friedrich, 2012; Dalsgaard et al. 2013).

An increasingly larger share of rainbow trout are produced in RAS, and future expansions of this industry rely on the availability of affordable feed combined with efficient removal of waste components, in-line as well as end-of-pipe, to ensure a high water quality and reduce the environmental footprint (Dalsgaard et al. 2013). To facilitate this development, the purpose of the present study was to resolve the effects of

gradually replacing fish meal with SBM on the utilization of the dietary nutrients by rainbow trout and the concurrent production of nutrient waste including nitrogen (N), phosphorous (P), and organic matter. In addition to determining the masses of waste produced, this also included determining the form (solid, dissolved and particulate) of the nutrient waste as this has a large impact on the water quality and which cleaning technologies to apply. Furthermore, the purpose of the study was to resolve if (and how) SBM in the diet affects the single sludge denitrification potential of the solid waste evaluated in terms of the masses and composition of VFA and ethanol that may be obtained via sludge hydrolysis and fermentation.

2.0 Materials and Methods

2.1 Fish and nutrient mass balance system

Juvenile rainbow trout (89.3 ± 11.7 g, mean ± SD) obtained from a local fish farm (Dybvadbro dambrug, Nibe, Denmark) were randomly distributed in 18, cylindro conical tanks mounted in a nutrient mass balance system (NMBS; described in Dalsgaard and Pedersen, 2011) at a stocking density of 28 fish per tank. Each tank with a volume of 188 L, was mounted with a sedimentation column via a union valve, and was supplied with tap water (11^{o}C) at a flow-through rate of 40 L/h. A photoperiod of 15 h light: 9 h dark was maintained throughout the study.

2.2 Experimental design and diets

A single factor experiment was performed including five experimental diets (Table 1) with increasing concentrations (10, 20, 30, 40 and 50%) of solvent extracted, toasted, high-protein (48%) SBM at the expense of FM. The diets were designed to be iso-nitrogenous with an anticipated, digestible protein level of 360 g/kg. Wheat was increasingly replaced by fish oil to balance the anticipated, digestible energy level at 18 MJ/kg, expecting a DP:DE ratio of 20 g/MJ in all diets. Methionine was supplemented to diets containing 30% SBM or more to balance the essential amino acid composition. A diet with FM as the sole protein source and an anticipated DP:DE ratio of 19 was included in the study as well. The diet was included to verify that the fish in the study performed as expected if fed a high performance, commercial like diet, and to enable a comparison with a previous study on the effects of different dietary DP:DE ratios on the production of VFA in the hydrolyzed and fermented sludge (Letelier-Gordo et al., 2015).

The experimental diets were formulated and produced by Biomar A/S, Denmark. They were extruded as 3 mm pellets and each diet was fed to triplicate tanks throughout the study. The fish were acclimatized to the diets and the tanks for three weeks followed by: i) a feeding trial for determining the apparent nutrient digestibility (9 days); and ii) a waste characterization trial (6 days).

The fish were fed restrictively throughout the study. Furthermore, the diets were fed iso-nitrogenous and iso-energetically by adjusting the daily rations relatively to the diet with the lowest, analyzed protein content (SBM_{10}; Table 1), and simultaneously adjusting the anticipated FCR relatively to the treatment group with the highest FCR during the acclimation period (SBM_{10}). As a consequence, the fish were fed 1.3% of the tank biomass of the SBM diets and 1.1% of the FM diet during the nutrient digestibility trial. The rations were reduced to 1 and 0.9% for the SBM and FM diets, respectively, during the waste characterization trial to avoid feed waste.

2.3 Fish performance, nutrient digestibility and retention

The fish were weighed individually at the start (t_0) of the nutrient digestibility trial and 8 fish from each tank were euthanized and stored at -20 ^{o}C until further carcass analysis. The daily ration was split in half and fed during 20 min at 10:00 am and 14:00 pm using automatic feeders. Union valves connecting the sedimentation columns to the tanks were closed during feeding to prevent feed waste from contaminating the feces collectors, and any feed waste was registered and enumerated immediately following each feeding event. The fish were weighed again at the end of the 9 days feeding trial (t_i), and the specific growth rate (SGR, % d^{-1}) was calculated using the equation (Hopkins, 1992): $SGR = Ln(W(t_i)/W(t_0))/(t_i-t_0) \times 100$; where $W(t_i)$ and $W(t_0)$ refer to the biomass at the end (t_i) and start (t_0) of the evaluation period. The corresponding FCR (g/g) was calculated as: FCR = feed consumed (t_i-t_0)/biomass gain (t_i-t_0).

All settleable fecal solids (sludge) produced during the 9 days were continuously collected in sedimentation columns enclosed in Styrofoam insulated containers with ice water to maintain the collected sludge at 0 ^{o}C. The proximate composition of pooled sludge samples (tank-basis) from feeding days 4-6 and 7-9 was analyzed as described in section 2.5, and apparent digestibility coefficients (ADC, %) were calculated following Talbot (1985) as: $ADC_i = ((consumed_i\text{-}excreted_i)/consumed_i)*100$; where i refers to the mass of crude protein, lipids, nitrogen free extract (NFE), ash, total phosphorous (TP) or gross energy consumed or excreted in the feces (i.e., recovered in the sludge).

Fish were sampled for carcass analysis at the start of the digestibility trial and end of the waste characterization trial, and the retention of digestible total nitrogen (TN), TP and gross energy was calculated as: $X_{retained} = (X(t_i) - X(t_0))/X_{digested}\ (t_i\text{-}t_0) \times 100$; where $X(t_i)$ and $X(t_0)$ refer to the content of TN, TP or gross energy in the fish biomass at the end (t_i) and start (t_0) of the trial and $X_{digested}$ (t_i-t_0) is the amount of consumed TN, TP or gross energy digested by the fish in the period.

2.4 Waste production and characterization

2.4.1 Solid waste for fermentation

The fish were fed a fixed ration during the waste characterization trial (1 and 0.9% of the SBM and FM diets, respectively) in order to relate the waste produced to the feed consumed. All the feed was fed during 20 min at 10:00 am, and the sludge produced in each tank was collected continuously during 4 consecutive days in the cooled sedimentation columns as explained in section 2.3. The collected sludge was pooled (tank-basis) and transferred to 1L Blue Cap bottles (SCHOTT Duran, Germany) serving as anaerobic batch reactors. The reactors were kept at room temperature (20 ± 2^{o}C) with continuous magnetic stirring at 200 rpm (Big Squid, IKA, Germany). The bottles were sealed with screw caps with two ports for sampling purposes (cap_GL, Duran Group, Germany), designed to avoid potential oxygen interference. Nitrogen gas was purged for 5 min into each bottle to ensure equal anaerobic conditions in each batch before starting a 7 days fermentation period. Samples of 50 mL were obtained at day 0 and frozen for latter nutrient characterization analysis including the total chemical oxygen demand (TCOD), total Kjeldahl nitrogen (TKN), lipids, and ash. In addition, samples of 30 mL were taken daily for analysis of the VFA composition and ethanol. The daily samples were centrifuged at 4500 rpm for 15 min at 0 ^{o}C immediately after acquisition and filtered through 0.2 µm syringe filters (Filtropour S, Sarstedt, Germany). The filtered filtered samples

were subsequently preserved by adding 1% v/v, 4 M sulfuric acid (H_2SO_4, Merck Millipore, Germany) and maintained at 4°C until analysis.

2.4.2 Dissolved waste

The masses of dissolved waste excreted by the fish to the water were determined by the end of the solid waste characterization trial by turning each tank into a closed-circuit reactor for 24 h and obtaining water samples at time 0 and 24 h. Subsamples for dissolved nitrogen (TN_{DISS}), total ammonia nitrogen (TAN), urea-N, NO_2N, NO_3N, and ortho-P analyses were immediately filtered through 0.2 µm syringe filters (Filtropour S, Sarstedt, Germany), while other subsamples were filtered through 0.45 µm mixed cellulose ester filters (Whatman, GE Healthcare, UK) prior to the analysis of dissolved COD (COD_{DISS}) and the dissolved, 5-day biological oxygen demand ($BOD_{5\text{-}DISS}$). Unfiltered subsamples for TP and TCOD analyses were conserved until analysis by adding 1% v/v 4 M sulfuric acid (H_2SO_4, Merck KGaA, Darmstadt, Germany). Samples for BOD_5 analyses ($TBOD_5$ and $BOD_{5\text{-}DISS}$) were analyzed right away while all other samples, including unfiltered samples for total nitrogen (TN), were stored at 4 °C until analysis (within 7 days at the most).

Sludge produced during the 24 h was sampled as described in section 2.3 and analyzed for TCOD and total BOD_5 ($TBOD_5$) as described in section 2.5.

Dissolved oxygen in the tanks was kept above 70% by aerating the water, simultaneously ensuring that the water was thoroughly mixed. Temperature increments were minimized by placing a polyvinyl chloride (PVC) pipe with cooling elements, which were regularly replaced, in the middle of each tank. As a result, the temperature was kept at 11 ± 0.5 °C. The trial was terminated after the last water sampling, and the fish were euthanized and stored at -20 °C until analysis for TN, TP and gross energy.

2.5 Chemical analysis

All chemical analyses were carried out in duplicate. Diet samples were ground using a Krups Speedy Pro homogenizer prior to the analysis of dry matter (DM) and ash (NMKL 23, 1991), TKN (ISO 5983-2, 2005 (crude protein = 6.25·Total Kjeldahl N)), lipid (Bligh and Dyer, 1959), and TP (ISO 6491, 1998). Nitrogen free extract (NFE) was determined indirectly as: NFE = DM – ash – lipid – protein. Gross energy was measured using a bomb calorimeter (IKA-Calorimeter C7000, IKA Analysentechnik, Heitersheim, Germany) after drying for 48 h at 60°C. Fecal samples from the digestibility trial were thawed and prepared using an Ultra Turrax homogenizer before analysis as described for the diets.

Total COD in sludge samples from the waste characterization trial was analyzed according to ISO 6060 (1989) using digestion vials (LCK 914, Hach Lange, Germany). The VFA composition in the filtered, daily samples from the sludge fermentation reactors was determined using a Perkin Elmer®Flexar® FX-15 UHPLC system fitted with a Flexar UV/Vis detector (PerkinElmer, USA). Separation was achieved using an ion exclusion column (Aminex HPX-87H 9µm, 300 mm x 7.8 mm, Biorad, USA) installed subsequently to a guard column (Micro-Guard Cation H cartridge, Biorad, USA). The mobile phase (0.005 M H_2SO_4) was run at a flow rate of 0.7 ml/min for a total of 60 min at 55 °C, and the detector was operated at 210 nm. Identified VFAs were quantified using individual standard curves ranging from 31 to 200 mg/L for acetic acid (HAc), 15.6 to 1000.0 mg/L for propionic acid (HPro), and from 7.8 to 500.0 mg/L for formic acid (HFo), butyric acid (HBu), and valeric acid (HVa) (Sigma-Aldrich, Germany). Ethanol in the samples was analyzed using a Megazyme Ethanol Assay Procedure (K-ETOH 01/14, Megazyme international Ireland Ltd, Ireland) and measured at 340 nm using a HACH Lange spectrophotometer (DR2800, HACH Lange, Germany).

Total nitrogen in water samples from the 24 h waste accumulation period was determined according to ISO 7890-1 (1986) and ISO 11905-1 (1997), NO_3N was determined according to ISO 7890-1 (1986), and NO_2N according to DS 223 (1991). Total ammonia nitrogen and urea-N was determined using the method described in Larsen et al. (2015), which allows for simultaneous analysis of both parameters. Total phosphorous and ortho-P were determined according to ISO 6878 (2004). The 5-days biological oxygen demand of the filtered and non-filtered water samples was analyzed according to ISO 5815-2 (2003) with allylthiourea (ATU), while COD was analyzed according to ISO 6060 (1989) using digestion vials (LCK 114, Hach Lange, Germany).

The 5-days biological oxygen demand of the sludge collected during the 24 h accumulation period was analyzed following ISO 5815-1 (2003) including ATU, and with the additional modification that the samples were homogenized using an Ultra Turrax prior to measuring BOD_5 in homogenized (rather than undisturbed) subsamples.

Fish fed the same diet and sampled at the start of the digestibility trial were pooled prior to analysis while fish sampled at the end of the waste characterization trial were pooled on a tank-basis. The pooled carcass samples were autoclaved for 90 min (1 bar, 120°C) and homogenized using a hand blender (Braun Multiquick 3, Type 4162, Kronberg, Germany). Subsamples of the paste were subsequently analyzed for TKN, TP and gross energy as described for the diets.

2.6 Statistical analysis

To test for significant differences between the investigated processes the results obtained from the different dietary treatments were subjected to one-way ANOVA analysis followed by Holm-Sidak orTukey-Kramer multiple comparison of means test. The one-way Anova analysis was run on ranks in case the normality test (Shapiro-Wilk) failed, followed by a Tukey multiple comparison test if significant differences were found. Differences were considered significant at $P < 0.05$, and values are stated as the mean ± standard deviation (SD). The analysis was carried out using the SigmaPlot 13 (Systat Software Inc, San Jose, California, USA) and R version 3 (R Core Team, 2013) software.

3.0 Results

3.1 Diets, fish performance and nutrient utilization

The content of protein and lipids in the SBM diets generally increased with higher SBM inclusion levels (from 37.4 to 38.7%, and 17.3 to 20.9%, respectively) while the content of NFE fluctuated in a more random manner (29.6% on average). As a result, there was a slight increase in gross energy (from 20.3 to 22.0 MJ/kg) with more SBM in the diets (Table 1). In contrast, the level of phosphorous decreased as the inclusion level of SBM increased (from 1.4 to 0.9%). In comparison to the SBM diets, the FM diet contained more proteins and lipids and less carbohydrates, and the gross energy content was therefore higher as anticipated.

There was no mortality in the trial. Fish fed the FM diet performed significantly better in terms of SGR and FCR than any of the SBM treatment groups (1.43 *versus* 0.84-1.17, and 0.80 *versus* 1.12-1.40, respectively; table 2). Fish fed the SBM diets performed more or less similar until an inclusion level of 30% after which performance was depressed ($P < 0.05$).

The apparent digestibility coefficients for the different nutrients and dietary treatment groups are summarized in table 2. The apparent digestibility of protein was similar (88.6 - 89.8% including the FM diet) for all treatments groups until an SBM inclusion level of 30% above which it increased ($P < 0.05$) to 91.0-91.2%. In contrast, the ADC of lipids was highest in the FM treatment group (89.2 %; $P < 0.05$) and decreased progressively as the inclusion level of SBM increased from 85.9 % in SBM_{10} to 77.6% for fish fed SBM_{50}. A similar but slightly less evident trend was observed for the ACD of gross energy, which was highest ($P < 0.05$) for the FM diet (88.7%) and decreased with higher inclusion levels of SBM (from 82.4 to 80.2% in SBM_{10} and SBM_{50}, respectively). The apparent digestibility of NFE was higher ($P < 0.05$) for fish fed the FM diet (76.1 %) than the SBM diets, where it ranged between 64.3 - 66.4% except from SBM_{50} where it fell to 61.7% ($P < 0.05$). The apparent digestibility of phosphorous showed the clearest dietary treatment response, increasing with each inclusion level of SBM (from 47.5 to 60.5% in the FM and SBM_{50} treatment group, respectively; $P < 0.05$). The increase was accompanied by a more or less similar trend in the ADC of ash, which increased from 32.9% in the FM diet to 47.5% in the SBM_{50} diet.

The realized DP:DE ratios for the SBM diets deviated slightly from the anticipated ratio of 20 g/MJ, fluctuating between 19.5 to 20.4 g/MJ. For the FM diet, the realized ratio was slightly higher than anticipated, i.e., 19.3 vs. 19.0 g/MJ.

The fish retained significantly less of the digested nitrogen as the inclusion level of SBM increased (decreasing from 56.3 to 37.2% in FM and SBM_{50}, respectively) whereas there was an opposite, non-significant trend in the retention of digestible phosphorous (increasing from 55.7 to 74.1% in SBM_{10} and SBM_{50}, respectively; table 2). Digestible energy retention was highest in the FM treatment group (69.3%; $P < 0.05$), while it for the SBM treatment groups was highest in the SBM_{10} group (49.2%) and lowest in the SBM_{50} group (34.9%), however, the differences were not significant.

3.2 Nutrient waste

To normalize and compare the masses of nutrient waste produced between the dietary treatment groups, the results are expressed in units of waste produced per unit of fish produced (i.e., specific production). The waste produced by fish fed the FM diet is only included as a reference for the discussion and is not included in the statistical analysis except for the day 0 waste characterization analysis prior to the 7-days sludge fermentation period (Table 3).

3.2.1 Nitrogen

The majority of the nitrogen waste was recovered in the water and the share increased from 78 to 85% as the level of SBM in the diets increased (Table 4). The increase in the specific production was mainly due to significant increases in dissolved N components including especially TAN (increasing from 21.6 to 32.9 g/kg fish produced in SBM_{10} and SBM_{50}, respectively) and to a lesser extent urea-N (increasing from 3.2 to 4.2 g/kg fish produced in SBM_{10} and SBM_{50}, respectively). This was accompanied by a similar but non-significant trend in the specific production of particulate N, which was lowest in the SBM_{10} group and highest in the $SBM_{40\text{-}50}$ groups (2.7 and 7.0 g/kg fish produced, respectively). There were no differences in the measured masses of NO_2-N and NO_3-N between the groups, and there were no differences in the recovery of TN in the sludge.

3.2.2 Phosphorous

The majority of the phosphorous waste (88-93%) was recovered in the sludge, and the amount apparently decreased as the level of SBM in the diet increased (from 7.6 to 5.0 g/kg fish produced in SBM_{10} and SBM_{50}, respectively; $P < 0.05$; table 4). Except for the SBM_{10} treatment group there was no measurable excretion of ortho-P by the fish (Table 4). More or less all the phosphorous recovered in the water was on particulate form (0.37 – 1.00 g/kg fish produced), and the amount was significantly lower in treatment groups $SBM_{30\text{-}50}$ than in treatment groups $SBM_{10\text{-}20}$.

3.2.3 Organic matter

The specific amounts of organic matter waste measured as TCOD increased significantly both in the water column and in the sludge as the dietary level of SBM increased (from 85 to 201, and from 287 to 425 g/kg fish produced in water and sludge, respectively; table 4). At the same time, the relative share of TCOD waste in the water contra in the sludge also increased (from 23 to 32% in SBM_{10} and SBM_{50}, respectively). Most of the specific COD waste in the water was present on dissolved form in the SBM_{10} group (70%) while the specific production of particulate COD became increasingly larger as more SBM was included in the diets, and in the SBM_{50} treatment group COD_{PART} constituted 50% of the COD in the water.

The specific production of organic matter waste measured as $TBOD_5$ followed the same trends as TCOD but constituted in most cases less than half of that of the corresponding TCOD. The specific production of $TBOD_{5\_WATER}$ increased from 33 to 83 g/kg fish produced while the specific production of $TBOD_{5\_SLUDGE}$ increased from 120 to 217 g/kg fish produced as the level of SBM in the diets increased (Table 4). The specific amounts of particulate BOD_5 in the water were always higher than those of dissolved BOD_5, and the fraction increased with more dietary SBM (from 19 to 75 g/kg fish produced; $P < 0.05$) whereas the specific production of $BOD_{5\text{-}DISS}$ stayed more or less the same (8 - 19 g/kg fish produced).

3.3 Sludge fermentation products

The characteristics of the pooled feces collected during four consecutive days in the waste characterization trial are shown in table 3. The specific yield of TCOD in sludge from fish fed the FM diet (0.20 g/g fish produced) was significantly lower than in sludge from fish fed the SBM diets. The yield of TCOD in the latter groups increased in a more or less linear manner with more SBM in the diets, i.e., from 0.25 g/g fish produced in sludge from the SBM_{10} group to 0.37 g/g fish produced in sludge from the SBM_{50} group.

The patterns in TCOD were largely mirrored in the other nutrients measured in the sludge. The specific amount of proteins was consequently lowest in sludge from fish fed the FM diet (33.6 mg/g fish produced) while it was highest in sludge from fish fed the SBM_{50} diet (38.3 mg/g fish produced), however, the differences were not significant. For lipids, the specific amount was significantly lower in sludge from the FM and $SBM_{10\text{-}20}$ treatments (17.6 – 23.1 mg/g fish produced) compared to the SBM_{50} treatment (40.0 mg/g fish produced). The specific amount of NFE was also lowest in sludge from the FM treatment group (56.9 mg/g fish produced), while it increased in a more or less linear manner with more SBM in the diets, i.e., from 91.2 mg/g fish produced in sludge from the SBM_{10} group to 151.6 mg/g fish produced in sludge from the SBM_{50} group.

The total amounts of VFA and ethanol (readily available carbon; RAC) produced after 7 days of sludge hydrolysis and fermentation expressed relative to the amounts of TCOD in the sludge measured at day 0,

decreased from 18 to 9% as the level of SBM in the diets increased (Figure 1). Butyric acid was the main VFA produced in treatment groups SBM_{10-20} (61-65 %), and was gradually replaced by acetic acid and ethanol as the level of SBM in the diets increased (constituting 42 and 31%, respectively in sludge from the SBM_{50} treatment group). Propionic and formic acid was produced to a lesser extent in sludge from all treatment groups while small amounts of valeric acids were only measured in sludge from SBM_{10-30}.

The amount of RAC/TCOD in fermented sludge from the FM treatment groups was largely similar to that of the SBM_{10} group (Figure 1). In contrast, the degree of fermentation (VFA_sCOD/sCOD) and VFA composition diverged somewhat from the SBM groups comprising mainly acetic acid (36%), propionic acid (28%), butyric acid (15%) and valeric acid (20%), while almost no ethanol (1%) was measured after the 7 days of fermentation. Hence, the yields of valeric acid and ethanol were significantly different from those obtained in the SBM treatment groups, the yields of acetic acid was significantly different from that in the SBM_{10} group, and the yields of propionic acid was significantly different from that in fermented sludge from the $SBM_{20,40-50}$ groups.

4.0 Discussion

4.1 Nutrient utilization and waste production

Feeding increasing levels of SBM to the fish clearly affected both their performance and the masses of waste produced. Consistent with previous studies (Collins et al., 2012; Hua and Bureau, 2012), fish performance was depressed when the diets included more than 30% SBM. The decline in fish performance was paralleled by a reduction in the apparent digestibility of lipids while the ADC of proteins generally increased. Collins et al. (2013a) discussed that positive effects on protein digestibility observed in previous studies where FM was partly replaced with SBM was indicative of a poor quality fish meal. This was not supported here where fish fed the FM diet (same raw material in all diets) performed significantly better than fish fed the SBM diets despite that all diets were fed iso-nitrogenous and iso-energetically. The FM diet contained comparatively more protein from FM and lipid from fish oil and less carbohydrates from wheat than a proper control diet would have, and this probably explains some of the performance differences. It, however, also sustains that the FM applied was of good quality and that rainbow trout generally digest SBM-based protein equally well or even slightly better than FM-based protein.

The availability of protein (i.e., proximate dietary protein content · $ADC_{protein}$) in the FM diet was 379 g/kg feed, while it increased from 331 g/kg feed in the SBM_{10} diet to 353 g/kg feed in the SBM_{50} diet. The increase in protein availability in the SBM diets was accompanied by a decrease in digestible nitrogen retention and an increase in dissolved N waste per unit fish produced, comprising mainly of TAN and to a lesser extent urea-N. Fish do not excrete NO_2-N and NO_3-N and the small production probably derived from nitrification happening at the walls of the tanks and pipes. The results show that the fish for some reason were not able to efficiently utilize the increase in available protein for anabolic processes/growth. Following “Liebing’s Law of the Minimum”, growth is controlled by the availability of the most limiting factor, which in this case may have been an essential amino acid. The proximate amino acid content and digestibility of the diets was thus not measured, and it cannot be ruled out that the availability of one or more essential amino acids was negatively affected by the SBM inclusion level. If that was so, the fish would have been forced to catabolize excess amino acids and excrete the nitrogen, and the effect would not be counteracted by the fact that the amino acid composition of the diets was apparently optimized.

An increase in dissolved nitrogen per unit fish produced would, however, also occur if the fish were limited in available energy and obliged to use assimilated protein to fuel basic metabolic processes. More SBM in the diets lead to a decrease in the apparent lipid digestibility which, however, was accounted for by a higher dietary lipid content. The measured DP:DE ratio in the diets was consequently more or less similar, and it therefore does not seem likely that the fish were energy limited and forced to catabolize protein to fuel metabolism. Furthermore, there was a tendency for the retention of digestible energy to decline with more SBM, indicating that the energy obtained was not efficiently utilized for growth despite that the fish, consistent with the DP:DE ratios, were not limited in available protein.

A poor growth performance of rainbow trout fed SBM has been ascribed to the presence of anti-nutritional factors (ANF; reviewed by Gatlin et al. 2007; Collins et al., 2013b). The high-protein, solvent extracted SBM used in the current study was toasted with the purpose to reduce the level of ANF. The process may, however, not have been sufficient to remove or reduce the level of especially saponins which, according to Collins et al. (2013b), are particularly problematic in SBM. Saponins are toxic to most cold-blooded animals including fish and may cause haemolysis of erythrocytes. Furthermore, saponins have been observed to cause damages to the intestinal mucosa of salmonids (reviewed by Francis et al., 2002; and by Sparg et al., 2004). Hence, rather than nutrient limitations and warranting further studies it may have been that the increase in dissolved N per unit fish produced reflects a toxic response coupling to an increase in the uptake of saponins or some other ANF.

Another well-known ANF in SBM is phytic acid, which may indirectly affect the performance of fish (Refstie et al., 1999; Francis et al., 2001; Gatlin et al., 2007) and the production of phosphorous waste (Dalsgaard et al., 2008). Most of the phosphorous in SBM is stored as phytic acid and is largely unavailable to the fish because they lack the enzyme necessary to break down the compound and release the phosphorous. Hence, even though the ADC of phosphorous in the current study increased significantly with more SBM the fish probably became increasingly more P limited because the availability of phosphorous (primarily deriving from dietary FM) concurrently declined. This would explain that there were no measurable, specific production of ortho-P by fish fed more than 10% SBM, and that the retention of digestible P increased significantly with more SBM in the diet. According to a previous study, there is a breakpoint value of 5.56 g available P/kg dry feed below which juvenile rainbow trout do not excrete ortho-P whereas they excrete ortho-P in a linearly increasing manner above the threshold (Dalsgaard and Pedersen, 2011). Fish fed the 10% SBM diet were above this threshold (10.80 g available P kg^{-1} dry feed standardized to a FCR of 1) while fish fed more than 10% SBM were progressively below the threshold (declining from 5.35 to 4.15 g available P kg^{-1} dry feed standardized to a FCR of 1). Fish can grow for a certain period on P-limited diets by utilizing their body stores without concurrent reductions in growth (Hardy et al., 1993; Sugiura et al., 2000). The final carcass P content of the fish in the current study was 0.442% in fish fed SBM_{10} and 0.435-0.439% in fish fed SBM_{20} - SBM_{50} fish, and although the values were not significantly different they sustain that the growth of fish fed more than 10% SBM was negatively affected by the availability of dietary phosphorous.

Increasing the dietary inclusion level of SBM had very large effects on the specific production of organic matter waste measured both in terms of COD and BOD_5. The significant increase in the production of organic matter waste in the sludge (Table 3) derived primarily from the increasing amounts of indigested lipids deduced from the changes in the apparent digestibility of the different dietary nutrients (Table 2). Hence, lipids are much richer in energy than proteins and carbohydrates (39.57 vs. 23.66 and 17.17 kJ/g, respectively; Jobling, 1994), and it requires therefore far more oxygen to degrade one unit of undigested

lipids recovered in the fish feces than one unit of undigested proteins or carbohydrates (Dalsgaard and Pedersen, 2011).

Soybean meal in the diet has been shown to slow down the sinking speed of rainbow trout feces (Ogunkoya et al., 2006), and this may partly explain the increases in particulate and dissolved organic matter. These latter fractions derive mainly from the breakdown of non-settled feces, and in addition to sinking speed SBM probably also reduced the cohesiveness of the feces, accelerating the disintegration of fecal particles before they settled out of the water column. This is supported by the increasingly larger share of the specific TCOD production being measured in the water compared to sludge, and the increasingly larger share of particulate vs. dissolved COD as more SBM was included in the diets. Furthermore, the specific production of $BOD_{5\text{-}DISS}$ stayed more or less the same while that of $BOD_{5\text{-}PART}$ increased, sustaining that increasingly more of the organic matter in the water derived from undigested (hard-to-degrade) nutrients with low solubility rather than from for example slime, small peptides and other unidentified organic compounds excreted from the fish.

In contrast to the production of particulate organic matter, the breakup and disintegration of feces was not accompanied by similar increases in the specific production of particulate N and P. For particulate N this related to the fact that the increase in apparent protein digestibility (and consequently less nitrogen in the feces) by fish fed increasingly more SBM was counteracted by a poorer feed conversion ratio. For phosphorous, the decrease in particulate P waste when fish were fed more than 20% SBM was due to significantly more phosphorous being assimilated by the fish rather than egested. Combined with the results on ortho-P this illustrates that there is a tight coupling between dietary P levels and availability, and the production of solid and dissolved P waste. Hence, rainbow trout seem to be enzymatically able to regulate the uptake of P deriving from fish meal depending on their needs. If fed in excess of requirements, the assimilation of dietary P is seemingly regulated in the gut and much of the digested P is egested rather than assimilated. If fed below requirements, the assimilation of P across the gut is improved and the excretion of ortho-P is reduced. Along these lines, a previous study showed that adding exogenous phytase to a rainbow trout diet containing plant meals in addition to FM presumably probably put this feed-back regulation system partly out of control. Hence, improving the availability of plant-based P when the availability of P from FM was already sufficient resulted in a large increase in the excretion of ortho-P, in effect transforming the P waste from being on solid form to dissolved form (Dalsgaard et al., 2008).

4.2 Waste nutrient treatment potential

The specific masses of $TCOD_{SLUDGE}$ in feces from fish fed the SBM diets were 1.25-1.85 times higher than that in sludge from fish fed the FM diet, indicating that sludge from fish fed the SBM diets had a theoretically higher capacity for biological nutrient removal than sludge from fish fed the FM diet. However, the fermentation degree (expressed as RAC/TCOD) of the sludge from the SBM treatments groups decreased as more SBM was included in the diets (Figure 1), meaning that less amounts of readily available carbon (VFA and ethanol) were produced in the fermentation process. Seven-days fermented sludge from the SBM_{10} treatment group had thus a similar potential for biological nutrient removal as similarly fermented sludge from the FM treatment group, whereas the potential declined with more SBM in the diet. The potential was thus lowest in sludge from the SBM_{50} treatment despite that sludge from this group contained the highest specific yield of TCOD.

Meriac et al. (2013) investigated the effect of dietary carbohydrate on the recovery and degradability of fecal waste from rainbow trout and found that comparatively more TCOD was produced when fish were fed non-starch polysaccharide diets compared starch based diets, while the biodegradability of the organic matter was significantly reduced. The authors speculated that the lower degradability of the COD (measured as BOD_{10}/COD) was due to poorly degradable lignocellulosic compounds. A similar theory may also partly explain the observed tendencies in the current study where the apparent NFE digestibility was lowest, and consequently the NFE content in the sludge was highest, in sludge deriving from the SBM_{50} treatment group. Furthermore, as more SBM was included in the diets the inclusion of wheat was similarly reduced, and the sludge presumable contained increasingly higher amounts of non-starch polysaccharides (NSP) deriving from SBM, which may have reduced the fermentation potential.

Another factor that might have affected the production of VFA and ethanol is the amounts of undigested lipids in the sludge. It has been reported that hydrolysis of lipids will not occur without the presence of methanogenic bacteria that can keep pH above acidic conditions and VFA levels at non-toxic concentrations (Zeeman and Sanders, 2001). This, possibly in combination with the content and composition of NFE, may potentially explain the observed reductions in the production of RAC as the level of lipids in the sludge increased with more SBM in the diet.

In addition to the total masses on readily available carbon, the apparent digestibility of protein, lipids and NFE presumably also affected the composition and masses of different VFA and ethanol produced in the fermented sludge (Figure 1). Large amounts of butyric acid were produced in sludge deriving from diets with the lowest amounts of SBM while the opposite was true for ethanol. Both butyric acid and ethanol can be produced from sugars and amino acids (Batstone et al., 2002). In accordance with this and with the apparent digestibility measurement it may be speculated that butyric acid measured in the sludge from fish fed the lowest amounts of SBM primarily derived from undigested proteins, whereas the increasing amounts of ethanol observed in sludge from diets with more SBM primarily derived from undigested NFE.

The different quantities and types of organic acids and ethanol that are produced and consequently are available for biological waste treatment are important for the nutrient removal process (N and P) in terms of carbon masses required, speed of the process, and bacteria produced (Akunna et al., 1993; Yatong, 1996; Lee and Welander, 1996). Using the different masses and types of organic acids and ethanol produced following the 7 days of fermentation it can be estimated that the fermented sludge deriving from $SBM_{10\text{-}40}$ had the potential to remove between 74-48% (decreasing as SBM inclusion increased) of the total N excreted by the fish. This is similar or slightly higher than similar calculations for the FM treatment (48%) and reflects that the denitrification potential responds to the types of carbon sources produced. Hence, bacteria able to use butyric acid and ethanol for denitrification, produced in high amounts in the SBM-based treatments, require a lower C:N ratio to run the process compared to bacteria fueled by valeric and acetic acid, which were produced in comparatively large amounts in the fermented sludge from the FM treatment (Yatong, 1996).

In the same manner, the phosphorous removal potential under enhance biological phosphorous removal (EBPR) is also affected by the type of carbon source produced. Abu-ghararah and Randall (1991) described that the amount of phosphorous removed per unit of COD decreased as the number of carbon atoms in the organic acids increased. This means that acetic acid and propionic acids are more efficient for P removal (better C:P ratio) than for example valeric acid which was produced in high amounts in the FM treatment group. Therefore the FM dietary group sowed a reduced capacity for phosphorous removal as compared to

468 the SBM dietary treatments which are able to remove between 19-23% of the TP produced while FM has
469 the potential to remove 14% of the TP produced.

470 **Conclusions**

471 The present study clearly showed that modifying the composition of a rainbow trout diet affects not only
472 the performance of the fish but also the masses and forms of nutrient waste produced. Replacing more
473 than 30% of FM-based protein with SBM-based protein depresses the growth performance of the fish and
474 increased the specific production of especially organic matter waste both in terms of BOD_5 and COD.
475 Soybean meal apparently reduced the cohesiveness of the feces, which resulted in an increasingly larger
476 load of especially particulate organic matter in the water. Particles are difficult (cost-intensive) to remove
477 and are highly unwanted especially in recirculating aquaculture systems where they are believed to affect
478 the health and performance of the fish either directly by intruding the gills or indirectly by providing surface
479 area and nutrients for potentially pathogenic bacteria. While increasing levels of SBM in the diet had
480 negative effects on fish performance and waste production, it had positive effects on the denitrification
481 potential (i.e., N removal) of the fermented sludge (at least up to a dietary SBM inclusion level of 40%)
482 compared to the potential of sludge deriving from fish fed a FM diet. This basically responds to the capacity
483 of different organic compounds has on the denitrification process, in this particular case the lower C:N
484 required by ethanol and butyric acid. Under an overall level dietary SBM not only showed to be an
485 alternative to reduce the pressure on FM as feed ingredient but also the potential to produce a residual
486 resource from the waste as ethanol. Although for dietary SBM to become a real sustainable alternative for
487 aquaculture, improvements in the fish performance and deterioration of the water quality are required.

488 **Acknowledgments**

489 This research was funded by The Ministry of Food, Agriculture and Fisheries of Denmark and by the
490 European Union through The European Fisheries Fund (EFF). The technical skills and invaluable assistance
491 of Sarah Møller Nielsen, Brian Møller and Ulla Sproegel are highly appreciated.

492

493 **Table 1.** Ingredients and gross composition on the experimental diets

Diet	FM	SPC_{10}	SPC_{20}	SPC_{30}	SPC_{40}	SPC_{50}
Ingredients (%)						
Fish meal [1]	58.7	44.4	37.4	31.1	24.7	18.29
Soya Cake 48 Hi Pro Solvent Extr.	0.00	10.0	20.0	30.0	40.0	50.0
Wheat	23.6	36.7	35.0	30.0	24.0	18.5
Fish oil	18.6	11.7	12.5	14.3	16.1	18.0
Methionine	0	0	0	0.02	0.10	0.17
Vitamins & minerals [2]	0.30	0.30	0.30	0.30	0.30	0.30
Proximate composition (%) [3]						
Dry Matter	93.7	92.5	93.6	94.4	93.8	93.7
Protein	42.5	37.4	37.5	37.5	38.0	38.7
Lipid	25.9	17.8	17.3	19.9	20.5	20.9

Ash	9.9	8.0	7.6	7.1	6.7	6.3
Phosphorous	1.7	1.4	1.2	1.1	1.0	0.9
NFE (nitrogen free extracts) [4]	15.4	29.3	31.2	29.9	28.6	28.8
Gross energy (MJ/kg)	*22.1*	*20.3*	*20.4*	*21.1*	*21.3*	*21.6*

[1] SA 68 superprime Perú, South America (68% protein).
[2] Premix Dk 3, Biomar A/S Denmark.
[3] Analyzed as described in Dalsgaard and Pedersen (2011).
[4] NFE calculated as: dry matter – protein –lipid – ash.

Table 2. Fish performance, apparent nutrient digestibility coefficients (ADC), and digestible nutrient and energy retention by the different dietary treatment groups (mean ± SD, n = 3) [1]

Diet	FM	SBM_{10}	SBM_{20}	SBM_{30}	SBM_{40}	SBM_{50}
Fish Performance						
SGR (% d^{-1})	$1.43^{a} \pm 0.04$	$1.17^{b} \pm 0.05$	$1.03^{bc} \pm 0.04$	$1.14^{bc} \pm 0.00$	$0.99^{cd} \pm 0.06$	$0.84^{d} \pm 0.12$
FCR	$0.80^{a} \pm 0.02$	$1.12^{b} \pm 0.04$	$1.25^{c} \pm 0.04$	$1.15^{bc} \pm 0.01$	$1.26^{c} \pm 0.02$	$1.40^{d} \pm 0.11$
ADC (%)						
Dry matter	$81.4^{a} \pm 0.8$	$76.4^{bc} \pm 0.3$	$75.2^{b} \pm 1.3$	$77.1^{c} \pm 0.6$	$77.2^{c} \pm 0.7$	$76.4^{c} \pm 1.9$
Crude protein	$89.2^{ab} \pm 0.4$	$88.6^{ab} \pm 0.4$	$88.6^{a} \pm 0.7$	$89.8^{b} \pm 0.4$	$91.0^{c} \pm 0.8$	$91.2^{c} \pm 1.1$
Crude lipid	$90.3^{a} \pm 1.7$	$85.9^{b} \pm 2.1$	$81.7^{c} \pm 2.4$	$81.5^{c} \pm 1.8$	$78.6^{cd} \pm 1.0$	$77.6^{d} \pm 3.3$
NFE	$76.1^{a} \pm 1.3$	$65.9^{b} \pm 0.4$	$64.3^{bc} \pm 2.2$	$66.4^{b} \pm 0.8$	$65.0^{b} \pm 1.8$	$61.7^{c} \pm 2.9$
TP	$47.5^{a} \pm 2.3$	$49.9^{b} \pm 0.4$	$52.2^{c} \pm 0.7$	$54.7^{d} \pm 1.6$	$58.1^{e} \pm 1.7$	$60.5^{f} \pm 2.0$
Ash	$32.9^{a} \pm 2.4$	$36.4^{ab} \pm 1.1$	$38.8^{b} \pm 1.8$	$43.0^{cd} \pm 2.4$	$46.6^{de} \pm 2.3$	$47.5^{e} \pm 3.7$
Gross energy	$88.7^{a} \pm 0.8$	$82.4^{b} \pm 0.8$	$80.3^{c} \pm 1.4$	$81.7^{bc} \pm 1.0$	$80.9^{bc} \pm 0.7$	$80.2^{c} \pm 2.0$
DP:DE (g/MJ)	$19.3^{a} \pm 0.2$	$19.8^{ab} \pm 0.3$	$20.2^{b} \pm 0.2$	$19.5^{ac} \pm 0.1$	$20.1^{bc} \pm 0.0$	$20.4^{b} \pm 0.3$
Retention (%)						
Digestible TN	$56.3^{a} \pm 2.0$	$55.9^{a} \pm 0.8$	$48.1^{b} \pm 0.6$	$45.9^{b} \pm 4.2$	$43.7^{b} \pm 2.4$	$37.2^{c} \pm 0.6$
Digestible TP	$68.0^{a} \pm 5.9$	$55.7^{a} \pm 5.3$	$55.3^{a} \pm 7.4$	$64.0^{a} \pm 13.3$	$68.3^{a} \pm 3.4$	$74.1^{a} \pm 11.0$
Digestible energy	$69.3^{a} \pm 2.1$	$49.2^{b} \pm 5.3$	$47.9^{b} \pm 5.6$	$38.8^{b} \pm 5.5$	$44.2^{b} \pm 1.8$	$34.9^{b} \pm 8.0$

[1]Values within rows not sharing a common superscript letter were significantly different (Holm-Sidak or Tukey-Kramer, $P < 0.05$).

Table 3. Characteristics (day 0) of settled feces produced by fish fed different diets and posteriorly used in the hydrolysis/fermentation batch study. Data are expressed as g masses produced/g or kg of fish produced (mean ± SD, n=3), and are based on daily sampling and subsequent pooling for four consecutive days[1]

Diet	FM	SBM_{10}	SBM_{20}	SBM_{30}	SBM_{40}	SBM_{50}
Dry Matter (g/g)	$0.13^{a} \pm 0.02$	$0.17^{ab} \pm 0.03$	$0.22^{bc} \pm 0.01$	$0.19^{bc} \pm 0.01$	$0.22^{bc} \pm 0.01$	$0.24^{c} \pm 0.03$
TCOD (g/g)	$0.20^{a} \pm 0.00$	$0.25^{b} \pm 0.01$	$0.31^{c} \pm 0.00$	$0.28^{d} \pm 0.00$	$0.32^{c} \pm 0.01$	$0.37^{e} \pm 0.01$
Protein (g/kg) [1]	$33.6^{a} \pm 2.9$	$37.8^{a} \pm 2.2$	$40.9^{a} \pm 3.8$	$36.1^{a} \pm 4.6$	$35.4^{a} \pm 3.1$	$38.3^{a} \pm 3.7$

Lipid (g/kg)	17.6^{a} ± 7.9	19.2^{a} ± 1.5	23.1^{a} ± 3.1	28.5^{ab} ± 4.9	34.8^{b} ± 7.4	40.0^{b} ± 4.7
NFE (g/kg) [2]	56.9^{c} ± 4.8	91.2^{ac} ± 30.7	140.2^{b} ± 8.8	115.6^{ab} ± 7.3	131.6^{b}± 4.7	151.6^{b} ± 20.9

[1] Protein was derived from TKN by multiplying by 6.25.
[2] NFE was calculated as NFE = Dry matter – protein – lipid – ash.

Table 4. Masses (g/kg fish produced) of nitrogen (N), phosphorous (P) and organic matter waste (COD and BOD_5) produced by the different dietary treatment groups (mean ± SD, n = 3). The FM treatment group is only included as a reference, and is not included in the statistical analysis [1)]

Diet	FM	SBM_{10}	SBM_{20}	SBM_{30}	SBM_{40}	SBM_{50}
N waste						
TN_{WATER}	21.8 ± 0.8	28.2^{a} ± 3.0	36.2^{b} ± 1.3	33.2^{ab} ± 1.9	37.9^{bc} ± 1.1	43.9^{c} ± 3.6
TN_{WATER_PART}	2.1 ± 1.6 [4]	2.7^{a} ± 2.4 [4]	5.0^{a} ± 1.4	3.9^{a} ± 0.9	7.0^{a} ± 0.6	7.0^{a} ± 2.8
TN_{WATER_DISS}	20.5 ± 2.1	26.8^{a} ± 2.9	30.8^{ab} ± 2.4	29.3^{ab} ± 0.9	30.4^{ab} ± 1.5	36.8^{b} ± 6.3
TAN_{WATER}	17.2 ± 1.0	21.6^{a} ± 2.1	27.0^{ab} ± 0.9	24.2^{a} ± 1.3	26.8^{ab} ± 1.9	32.9^{b} ± 4.6
Urea-N_{WATER}	2.6 ± 0.1	3.2^{a} ± 0.2	3.6^{ab} ± 0.2	3.5^{ab} ± 0.2	3.8^{ab} ± 0.2	4.2^{b} ± 0.4
NO_2N_{WATER}	1.1 ± 0.0	1.6^{a} ± 0.8	1.5^{a} ± 0.3	1.5^{a} ± 0.3	1.8^{a} ± 0.7	2.1^{a} ± 0.4
NO_3N_{WATER}	0.9 ± 0.4	1.5^{a} ± 0.2	1.2^{a} ± 1.0	1.2^{a} ± 0.5	0.9^{a} ± 0.2	0.7^{a} ± 0.5
TN_{SLUDGE}	5.9 ± 0.3	7.6^{a} ± 0.5	8.6^{a} ± 0.6	7.0^{a} ± 0.2	6.9^{a} ± 0.5	7.7^{a} ± 1.1
P waste						
TP_{WATER}	0.85 ± 0.27	0.99^{a} ± 0.19	1.00^{a} ± 0.26	0.37^{b} ± 0.11	0.38^{b} ± 0.25	0.39^{b} ± 0.08
P_{WATER_PART}	0.38 ± 0.08	0.81^{ab} ± 0.17	1.00^{a} ± 0.26	0.37^{b} ± 0.11	0.38^{b} ± 0.25	0.39^{b} ± 0.08
Ortho-P_{WATER}	0.47 ± 0.23	0.18 ± 0.21	0 [3)]	0 [3)]	0 [3)]	0 [3)]
TP_{SLUDGE}	7.2 ± 0.5	7.6 ± 0.3^{a}	7.4 ± 0.3^{a}	5.8 ± 0.1^{b}	5.3 ± 0.2^{b}	5.0 ± 0.3^{b}
COD waste						
$TCOD_{WATER}$	59.2 ± 3.2	84.6^{a} ± 12.3	125.2^{ab} ± 17.1	137.0^{b} ± 13.9	148.5^{b} ± 17.6	201.3^{c} ± 25.0
COD_{WATER_PART}	6.2 ± 5.7 [4)]	25.3^{a} ± 13.0	50.6^{ab} ± 12.3	58.8^{ab} ± 19.4	64.8^{ab} ± 20.9	100.1^{b} ± 31.0
COD_{WATER_DISS}	48.4 ± 6.6	59.3^{a} ± 3.8	74.6^{ab} ± 5.9	78.1^{b} ± 10.2	83.8^{b} ± 4.4	101.2^{c} ± 6.2
$TCOD_{SLUDGE}$	136.9 ± 3.6	287.2^{a} ± 10.3	343.8^{ab} ± 26.6	327.2^{a} ± 11.0	361.8^{ab} ± 11.4	425.3^{b} ± 56.3
BOD_5 waste						
$TBOD_{5\_WATER}$	16.7 ± 3.5	32.5^{a} ± 4.5	43.9^{a} ± 9.3	57.9^{ab} ± 3.0	60.2^{ab} ± 3.3	83.2^{b} ± 24.1
$BOD_{5\_WATER\_PART}$	3.4 ± 0.3	19.4^{a} ± 3.0	24.7^{ab} ± 7.9	39.5^{ab} ± 4.3	47.8^{ab} ± 3.6	75.1^{b} ± 25.8
$BOD_{5\_WATER\_DISS}$	13.3 ± 3.3	13.2^{a} ± 1.8	19.1^{a} ± 2.7	18.4^{a} ± 4.2	12.5^{a} ± 6.5	8.0^{a} ± 1.7
$TBOD_{SLUDGE}$ [2]	65.5 ± 16.1	119.9^{a} ± 4.7	158.9^{b} ± 3.5	152.3^{ab} ± 19.7	185.1^{bc} ± 13.4	217.4^{c} ± 21.4

[1] Values within rows not sharing a common superscript letter were significantly different (Holm-Sidak or Tukey-Kramer, $P < 0.05$). The FM treatment group was not included in the statistical analysis.
[2] Analyzed following ISO 5815-1 (2003) but with the modification that samples were homogenized prior to measuring BOD_5 in homogenized (rather than undisturbed) subsamples.
[3] No measurable increase in ortho-P during the 24 h accumulation trial, and the value therefore set to zero. As an effect, all measured phosphorous in the water was assumed to be present in the particulate form.
[4] n = 2

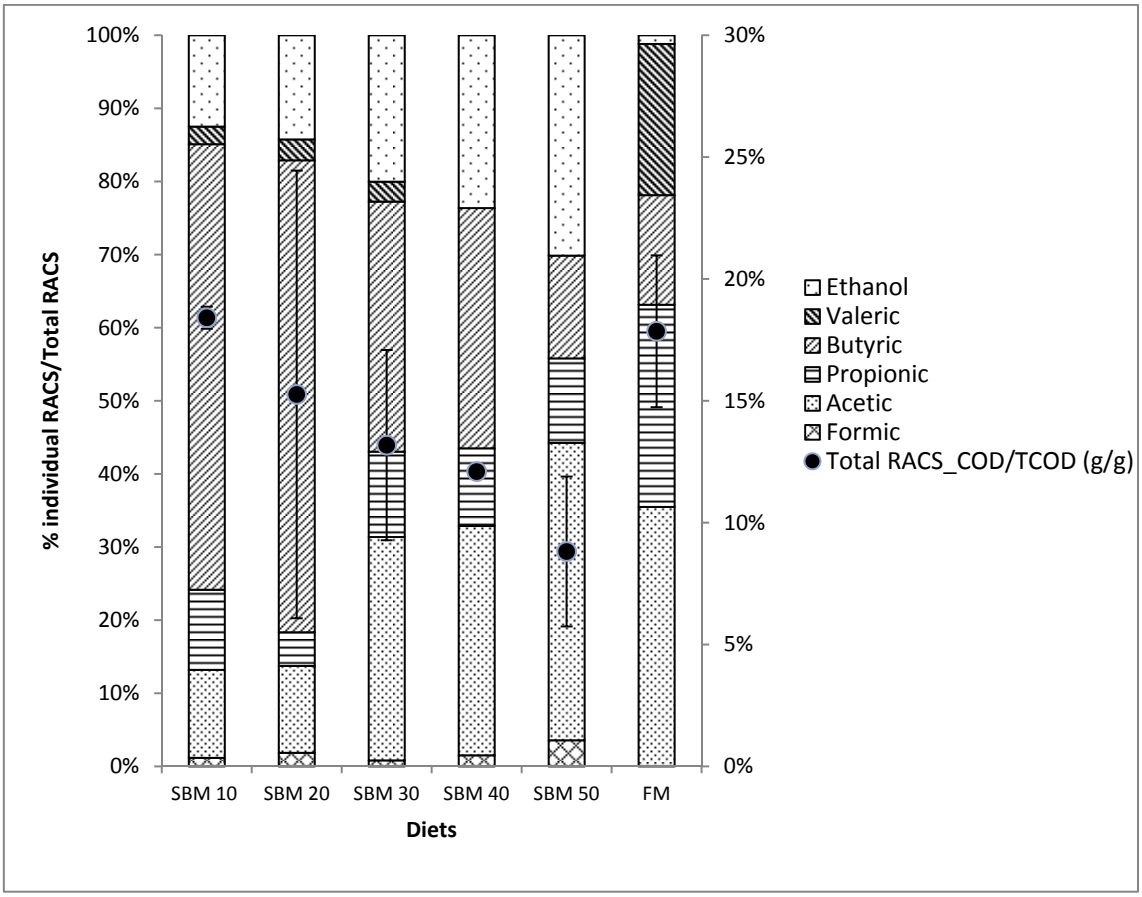


Figure 1. Composition of the readily available carbon (RACs) measured after hydrolyzing/fermenting the settled feces/sludge for 7 days deriving from the different dietary treatment groups. The average, relative composition of the RACS is shown on the left axis (n=3), while total yields (g RACS/g TCOD) are shown on the right axis (mean ± SD, n=3).

References

Abu-ghararah, Z. H., Randall, C. W. 1996. The effect of organic compounds on biological phosphorous removal. Water Science and technology 23, 585-594.

Akunna, J.C., Bizeau, C., Moletta, R., 1993. Nitrate and nitrite reductions with anaerobic sludge using various carbon sources: glucose, glycerol, acetic acid, lactic acid and methanol. Water Research 27 (8), 1303-1312.

Batstone, D.J., Keller, J., Angelidaki, I., Kalyuzhnyi, S.V., Pavlostathis, S.G., Rozzi, A., Sanders, W.T.M., Siegrist, H., Vvili, V.A., 2002. The IWA Anaerobic digestion model No 1 (ADM1). Water Science and Technology 45 (10), 65-73.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911-917.

Brinker, A., Reiter, R., 2011. Fish meal replacement by plant protein substitution and guar gum addition in trout feed, part I: effects on feed utilization and fish quality. Aquaculture 310, 350-360.

Brinker, A., Friedrich C. 2012.Fish meal replacement by plant protein substitution and guar gum addition in trout feed. Part II: Effects on faeces stability and rheology. Biorheology 49, 27-48

544 Bureau and Hua, 2010. Towards effective nutritional management of waste outputs in aquaculture, with
545 particular reference to salmonid aquaculture operations. Aquaculture Research 41, 777-792

546 Collins, S.A., Øverland, M., Skrede, A., Drew, M. 2013a. Effect of plant protein sources on growth rate in
547 salmonids: Meta-analysis of dietary inclusion of soybean, pea, canola/rapeseed meals and protein
548 concetrates. Aquaculture 400, 85-100.

549 Collins, S.A., Mansfield, G.S., Desai, A.R., Van Kessel, A.G., Hill, J.E., Drew, M.D. 2013b. Structural equation
550 modeling of antinutrients in rainbow trout diets and their impact on feed intake and growth. Aquaculture
551 416, 219-227.

552 Collins, S.A., Desai, A.R., Mansfield, G.S., Hill, J.E., Van Kessel, A.G., Drew, M.D. 2012. The effect of
553 increasing inclusion rates of soybean, pea and canola meals and their protein concentrates on the growth
554 of rainbow trout: Concepts in diet formulation and experimental design for ingredient evaluation.
555 Aquaculture 344, 90-99.

556 Dalsgaard, J., Pedersen, P.B. 2011. Solid and suspended/dissolved waste (N, P, O) from rainbow trout
557 (*Oncorhynchus mykiss*). Aquaculture 313, 92–99.

558 Dalsgaard, J., Ekmann, K.S., Pedersen, P.B., Verlhac, V. 2008. Effect of supplemented microbial phytase on
559 the performance and fate of dietary phosphorus in phosphorus deficient juvenile rainbow trout
560 (*Onchorynchus mykiss*). Aquaculture 286, 105-112.

561 Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K., Pedersen, P.B. 2013. Farming different
562 species in RAS in Nordic countries: Current status and future perspectives. Aquacultural Engineering 53, 2-
563 13.

564 FAO, 2016. Food and agriculture organization of the United Nations. Statistics Division.
565 http://faostat3.fao.org/. Accessed 9 October 2016.

566 Francis, G., Makkar, H.P.S., Becker, K., 2001. Antinutritional factors present in plant-derived alternate fish
567 feed ingredients and their effects in fish. Aquaculture 199, 197-227.

568 Francis, G., Kerem, Z., Makkar, H.P.S. Becker, K. 2002. The biological action of saponins in animal systems: a
569 review. British Journal of Nutrition 88, 578-605.

570 Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G.S.,
571 Krogdahl, A., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., Souza, E.J., Stone, D., Wilson, R.,
572 Wurtele, E., 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquacult.
573 Res. 38, 551-579

574 Hardy, R.W., 1996. Alternate protein sources for salmon and trout diets. Animal Feed Science and
575 Technology 59, 71-80.

576 Hardy, R.W., 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of
577 fishmeal. Aquaculture Research 41, 770–776.

578 Hardy, R.W., Fairgrieve, W.T., Scott, T.M., 1993. Periodic feeding of low-phosphorus diet and phosphorus
579 retention in rainbow trout (*Oncorhynchus mykiss*). In: Kaushik, S.J., Luquet, P. (Eds), Fish Nutrition in
580 Practice. IVth International Symposium on Fish Nutrition and Feeding, INRA Editions, Paris, France, pp. 403-
581 412.

582 Hopkins, K.D., 1992. Reporting fish growth: a review of the basics. J. World Aquacult. Soc. 23, 173-179.

583 Hua, K. and Bureau, D.P., 2012. Exploring the possibility of quantifying the effects of plant protein
584 ingredients in fish feeds using meta-analysis and nutritional model simulation-based approaches.
585 Aquaculture 356-357, 384-301

ISO 5815-1, 2003. Water quality - Determination of biochemical oxygen demand after n days (BODn) – Part 1: Dilution and seeding method. International Organization for Standardization, Geneva, Switzerland, 15 pp.

ISO 5815-2, 2003. Water quality - Determination of biochemical oxygen demand after n days (BODn) - Part 2: Method for undiluted samples. International Organization for Standardization, Geneva, Switzerland, 13 pp.

ISO 5983-2, 2005. Animal feeding stuffs - Determination of nitrogen content and calculation of crude protein content - Part 2: Block digestion/steam distillation method. International Organization for Standardization, Geneva, Switzerland, 14 pp.

ISO 6060, 1989. Water quality - Determination of the chemical oxygen demand. International Organization for Standardization, Geneva, Switzerland, 4 pp.

ISO 6491, 1998. Animal feeding stuffs - Determination of phosphorus content - spectrometric method. International Organization for Standardization, Geneva, Switzerland, 16 pp.

ISO 6878, 2004. Water quality – Determination of phosphorus – Ammonium molybdate spectrometric method. International Organization for Standardization, Geneva, Switzerland, 21 pp.

Jobling, M., 1994. Fish Bioenergetics, first ed. Chapman & Hall, London.

Klas, S., Mozes, N., Lahav, O. 2006. Development of a single-sludge denitrification method for nitrate removal from RAS effluents: Lab-scale results vs. model prediction. Aquaculture 259, 342-353

Larsen, B.K., Dalsgaard, J., Pedersen, P.B. 2015. An optimized and simplified method for analyzing urea and ammonia in freshwater aquaculture systems. Aquaculture Research 46, 1608-1618.

Lee, N., Welander, T., 1996. The effect of different carbon sources on respiratory denitrification in biological waste water. Journal of Fermentation and Bioengineering 83 (3), 277-285

Letelier-Gordo, C.O. Dalsgaard, J., Suhr, K., Ekmann, K.S., Pedersen, P.B., 2015. Reducing the dietary protein:energy (P:E) ratio changes solubilization and fermentation of rainbow trout (Oncorhynchus mykiss) faeces. Aquacultural Engineering. 66, 22-29.

Meriac, A., Eding, Ep. H., Schrama, J., Kamstra, A., Verreth, J.A.J. 2013. Dietary carbohydrate composition can change waste production and biofilter load in recirculating aquaculture systems. Aquaculture 420-421, 254-261.

NMKL 23, 1991. Moisture and ash. Gravimetric determination in meat and meat products, NMKL 23. Nordic Committee on Food Analysis, Oslo, Norway, 4 pp.

Ogunkoya, A.E., Page, G.I., Adewolu, M.A., Bureau, D.P., 2006. Dietary incorporation of soybean meal and exogenous enzyme cocktail can affect physical characteristics of faecal material egested by rainbow trout (*Oncorhynchus mykiss*). Aquaculture 254, 466-475.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Refstie, S., Svihus, B., Shearer, K.D., Storebakken, T., 1999. Nutrient digestibility in Atlantic salmon and broiler chickens related to viscosity and non-starch polysaccharide content in different soyabean products. Anim. Feed Sci. Technol. 79, 331-345.

Romarheim, O.H., Skrede, A., Gao, Y.L., Krogdahl, A., Denstadli, V., Lilleeng, E., Storebakken, T., 2006. Comparison of white flakes and toasted soybean meal partly replacing fish meal as protein source in extruded feed for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 256, 354-364.

Sparg, S., Light, M.E., van Staden, J. 2004. Biological activities and distribution of plant saponins. Journal of ethnopharmacology 94, 219-243.

628 Storebakken, T., Refstie, S., Ruyter, B., 2000. Soy products as fat and protein sources in fish feeds for
629 intensive aquaculture, in: Drackley, J.K. (Ed.), Soy in animal nutrition. Federation of Animal Science
630 Societies, Savoy, IL, pp. 127-170.

631 Sugiura, S.H., Dong, F.M., Hardy, R.W., 2000. A new approach to estimating the minimum dietary
632 requirement of phosphorus for large rainbow trout based on non fecal excretions of phosphorus and
633 nitrogen. J. Nutr. 130, 865-872.

634 Talbot, C. 1985. Laboratory methods in fish feeding and nutritional studies, in: Tytler, P., Calow P. (Eds.),
635 Fish energetics: New perspectives, Croom Helm Ltd., London, UK, pp. 125-154. ISBN 0-7099-3410-6.

636 USSEC, 2008. Soy protein concentrate for aquaculture feeds. Technical bulletin. USSEC. USSEC World
637 Headquarters. U.S. Soybean Export Council, St. Louis, USA, 11 pp.

638 Yang, Y.-H., Wang, Y.-Y., Lu, Y., Li, Q.-Z.Y. 2011. Effect of replacing fish meal with soybean meal on growth,
639 feed utilization and nitrogen and phosphorus excretion on rainbow trout (*Oncorhynchus mykiss*).
640 Aquaculture International 19(3), 405-419.

641 Yatong, X., 1996. Volatile fatty acids carbon source for biological denitrification. Journal Environmental
642 Science 8, 257-268.

643 Zeeman, G., Sanders, W. 2001. Potential of anaerobic digestion of complex waste (water). Water Science
644 and Technology 44, 115–122.

645

646

Paper IV (manuscript)

Applicability of internal carbon sources for a denitrification system on a low intensity brood stock Danish trout farm, a mass balance approach.

Abstract

The applicability of using internal carbon sources for denitrification on a low intensity brood stock Danish rainbow trout (Oncorhynchus mykkis) farm was evaluated. Organic waste from normal cleaning operation of the system (hatchery, sludge cones and biofilters) was used to feed a side stream fermenter (SSF) for producing dissolved carbon forms measured as soluble chemical oxygen demand (sCOD) volatile fatty acids (VFAs). The produced dissolved organic forms (sCOD and VFAs) were supplied to a denitrification reactor operated at three different flows (6, 18 and 54 m^3/d). The results showed that the SSF managed to enhance the quality of the organic waste for removing N (13.2% Total nitrogen (TN) and P (62% Total phosphorous (TP) at the end-of-pipe treatment, additionally the organic waste was also reduced (71.3% Total chemical oxygen demand (TCOD). However, the quality of the collected organic waste limited the performance of the system as a low degree of solubilization was found (2% gsCOD/gTCOD). It was calculated that 1 m^3 of the enhanced sludge in the SSF removes 92.2 g of NO_3^- N plus 381 g of oxygen on a daily basis. Thus, 27 m^3 of organic waste would be required to remove 2.5 Kg NO_3-N/d (amount required by the farm to comply with the environmental regulation) and 10.3 KgO_2/d from the incoming water to achieve anoxic conditions. The system showed to be an interesting alternative for end-of-pipe treatment, although carbon quantity and quality limits the maximal potential in this type of farm. Improvements in this respect should point at reducing the mass of oxygen entering the denitrification reactor, adopting a recycling flow within the denitrification reactor and improving the organic waste collection method.

1. Introduction

Environmental sustainability has become a key issue in aquaculture, particularly regarding source of feed ingredients, alterations of ecosystems and discharge of waste towards water receiving bodies (Martins et al., 2010; van Rijn, 2013). For an improved sustainability, aquaculture would need a profitable production decoupled from its ecological impacts. An increasing number of Danish freshwater farms have converted from traditional open flow through systems into Model-Trout-Farms (MTFs), incorporating a series of water treatment devices and water recycling operations. The technology has allowed Danish farmers to increase their production capacity under actual strict environmental regulations (Danish Ministry of environment, 2012).

The MTFs water treatment system includes particle removal devices, e.g., sludge cones and drum filters, and aerobic biological filters for converting NH_4^+ to NO_3^-, allowing the recirculation of 70-95% of the RAS effluent back to the production system. The non-recirculated flow is treated in constructed wetlands before being discharged into water receiving bodies such as lakes and rivers. A MTF can remove up to 75% of biochemical oxygen demand (BOD), 60% of TP and 11% TN discharged, this last parameter being one of the main limiting factors preventing the Danish (and European) industry from increasing its production (Jokumsen and Svendsen, 2010; Dalsgaard et al., 2013). To overcome this challenge, efforts are concentrated on developing cost effective technologies for removing TN from the recirculating aquaculture system (RAS) discharge water, with special emphasis on NO_3^-- N, which constitutes more than 80% of TN (Timmons et al., 2008; Diaz et al., 2012).

Denitrification processes at the end-of-pipe treatment in aquaculture systems gain more attention every day as improving the reduction of N discharged became a necessity for complying with environmental

regulations (Tal et al., 2006; Hamlin et al., 2008; van Rijn, 2013; Suhr et al., 2014). The addition of methanol and acetic acid is a frequent commercial practice to boost the process, showing good results in terms of stability and controllability (Henze et al., 2002; Ucisik and Henze, 2008; Hamlin et al., 2008).

Denitrifying bacteria can use a wide spectrum of carbon sources, classified as external (commercially obtained) or internal (produced within the system) (Henze et al., 2008). Applying external carbon sources for denitrification has been evaluated in RAS with the objective of increasing the water intensity of the system or improving temperature control. Otte and Rosenthal (1979) used glucose and methanol as carbon source for a denitrification reactor for eel culture achieving around 50% removal of nitrate, Suzuki et al. (2003) used methanol for a zero discharge eel culture system, reducing 90% of the nitrate accumulated in the rearing tank. Hamlin et al. (2008) evaluated four different carbon sources (methanol, acetic acid, molasses and a hydrolyzed starch) reaching denitrification rates of 670-800 g NO_3^--N/m^3 media/d, showing effective removal of nitrate to near zero concentrations. Adding external carbon sources to denitrification systems in aquaculture has proven to be an effective solution for controlling nitrate. However, the addition of external carbon sources increases the process operational costs and sludge production (Ucisik and Henze, 2008). Organic waste produced by the fish in RAS has shown to have potential for serving as an internal carbon source for denitrification (Jewel and Cummings, 1990; van Rijn et al., 2006; Tal et al., 2009; Suhr et al., 2014), but no further research fully explained its potential. Few data exists on the chemical composition, electron donating properties and biodegradability characteristics of the recoverable organic waste generated in a RAS (Klas et al., 2006; van Rijn et al., 2006). Quantifying the mass and type of the organic matter discharged from the RAS is of major importance in order to attempt any prediction on the capacity for using internal carbon sources for on-farm denitrification. The following study evaluated the applicability of a side stream fermenter (SSF) for improving the quality of internal carbon sources obtained from the backwash of a drum filter for applying on an end-of-pipe denitrification system in a low intensity brood stock Danish rainbow trout trout farm.

2. Materials and methods

2.1 The study site

The study was carried out in a low intensity (21.6 m^3/Kg feed) brood stock rainbow trout Danish farm located in the northern part of Denmark, comprising a hatchery and 10 earthen raceways for fish husbandry. The farm has an internal flow of 8208 m^3/d from which 6048 m^3/d are recirculated back to the fish tanks after being treated with sludge cones and a fixed bed biofilter. The non- recirculated flow (Q = 2160 m^3/d) is lead through a larger, fixed, end-of-pipe biofilter (81 m^3) with Bio-Blok® (200 m^2/m^3) before entering a small constructed wetland for final polishing.

2.2 The side stream fermenter (SSF)

To evaluate the applicability of a side stream fermenter (SSF) under commercial scale conditions, water from the backwash of a 60 μm drum filter (#1 in Figure 1) was used to supply organic waste to the SSF. The SSF consisted in 2 parallel cylindrical concrete tanks with an individual volume of 11.9 m^3 (#2 in Figure 1). The SSF were situated in parallel and worked alternatively in order to deliver a constant supply of organic waste through a distribution pump (#3 in Figure 1) into the denitrification reactor (#4 in Figure 1). The SSF was filled with organic waste derived from the cleaning water of the hatchery tanks (Letter A in Figure 1), the sludge cones (Letter B in Figure 1) and backwash of the two biofilters (Letter C and D in Figure 1). Water from the 81 m^3 fixed biofilter overflow (Letter D in Figure 1), was pumped into a moving bed denitrification reactor (#3 in Figure 1) which provided a constant supply of water containing NO_3^-.

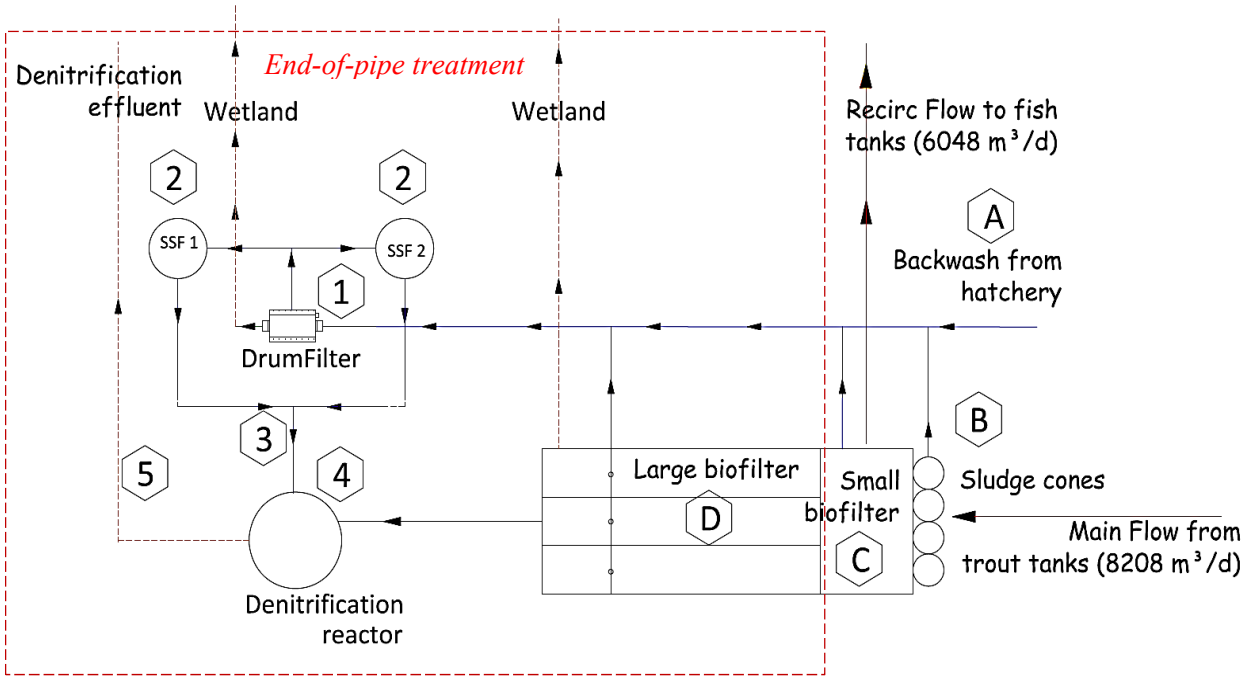


Fig. 1. Diagram of the end-of-pipe treatment (dashed lines: (1) drum filter (2) side stream fermenter (SSF) 11.9 m^3 each (3) distribution pump (4) denitrification reactor 20.3 m^3 moving bed (5) effluent from the denitrification reactor into the wetland. The organic waste sources for the SSF were obtained from: (A) backwash of hatchery (B) sludge cones (C) small biofilter 44 m^3 fixed bed (D) large biofilter 81 m^3 fixed bed.

Previously to start of the trial a flow characterization was developed in order to estimate the amount of carbon sources able to recover from a weekly cleaning routine of the treatment devices which posteriorly feed the SSF (Table 1).

Table 1. Flow values from the different sources feeding the sequence step batch reactors.

Flows	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday	Total flow
	(m^3/d)	(m^3/d)	(m^3/d)	(m^3/d)	(m^3/d)	(m^3/d)	(m^3/d)	
Hatchery	0.05	0.05	0.05	0.05	0.05	0.05	0.05	**0.35**
Sludge cones	0.09	0.09	0.09	0.09	0.09	0.09	0.09	**0.63**
Small Biofilter	0.43	0.43						**0.86**
Large Biofilter			1.54					**1.54**
Sub total	**0.57**	**0.57**	**1.68**	**0.14**	**0.14**	**0.14**	**0.14**	**3.38 m^3/week**

From the flow characterization an input of 3.38 m^3 of organic waste entering the SSF was recovered. For evaluation purposes and to standardize a constant input of flows and type of organic waste collected, the flow discharged from the SSF to denitrification reactor system was set to 0.48 m^3/d or a hydraulic retention time (HRT) of 7 days. Water was pumped from the SSF into the denitrification reactor in pulse mode delivering 1L of organic waste every three minutes, monitored daily using the volumetric method.

2.3 Characterization of the organic waste quality

To characterize the quality of the different types of organic waste feeding the SSF (hatchery, small biofilter, large biofilter and sludge cones), samples were individually drawn and characterized for their degradability in anoxic/anaerobic laboratory conditions. The individual organic waste samples were kept in 1L reactors enclosed with Blue Cap bottles (SCHOTT Duran, Germany) for maintaining anoxic/anaerobic

conditions. The reactors were kept at room temperature (20 ± 2°C) with continuous magnetic stirring at 200 rpm (Big Squid, IKA, Germany). The bottles were sealed with screw caps with two ports for sampling purposes (cap_GL, Duran Group, Germany), to avoid potential oxygen interference. Nitrogen gas was purged for 3 min into each bottle to ensure equal anoxic conditions in each batch before starting the degradability trial.

2.4 The denitrification reactor

The denitrification reactor was a moving bed with a volume of 20.3 m^3 and was filled 50% with RK BioElements (750 m^2/m^3). The denitrification reactor was fed with two waste streams 1) water pumped from the 81 m^3 fixed biofilter (Letter D in Figure 1) majorly containing NO_3^--N as N form and 2) water discharged from the SSF containing the collected internal carbon sources (#4 in Figure 1). The two waste streams merged into a main pipe at the central bottom part of the denitrification reactor. The media in the denitrification reactor was mixed periodically using stain steel plaques connected to an axial rotor. The effluent of the denitrification reactor was discharged into a small wetland (#5 in Figure 1).

2.5 The trials and sampling procedure

2.5.1 The farm trial

According to the amount of organic waste able to recover from the cleaning of the hatchery, sludge cones and biofilter, the denitrification reactor was set to operate under three different flows 6, 18 and 54 m^3/d with a hydraulic retention time (HRT) of 3.3, 1.1 and 0.4 days respectively. In parallel the SSF discharged a continuous flow of 0.48 m^3/d with a HRT of 7 days. The flows entering the denitrification reactor were daily measured using a portable flow meter (Portaflow 204 Plus, Micronics, UK), while the flows discharged from the SSF were measured using the volumetric method.

The trial lasted 42 days where each selected flow in the denitrification reactor (6, 18 and 54 m^3/d) was evaluated during 14 days. Samples from each organic waste type (hatchery, sludge cones and biofilters) were obtain weekly accordingly to the cleaning protocol of the devices (Table 1) and transfer to laboratory to characterize their properties for anoxic/anaerobic degradation. Simultaneously, 24 h pooled samples with a sampling frequency of an hour were taken every two days in: 1) the water pumped from the 81 m^3 fixed biofilter into the denitrification reactor (Leter D in Figure 1) 2) the water pumped from the SSF into the denitrification reactor (#3 in Figure 1) and 3) the water discharged from the denitrification reactor (#5 in Figure 1). All samples were taken with an automatic portable sampler (Glacier ISCO, Teledyne, USA) and refrigerated at 4°C before transferring them for laboratory analysis. TCOD, TP, total Kjeldahl Nitrogen (TKN), total ammonia nitrogen (TAN), phosphorus expressed as ortho-phosphate (PO_4^{3-}-P), nitrate (NO_3^--N), nitrite (NO_2^--N), VFAs, and sCOD were analyzed at the laboratory. Dissolved oxygen (DO), temperature and pH were measured hourly on site using a portable meter (Hach HQ40d, Hach Lange, Germany).

2.5.2 The laboratory trial

A characterization of the quality of each organic waste type (hatchery, sludge cones and biofilters) was evaluated during 7 days to simulate the HRT set in the SSF. Daily samples of 30 mL were obtained for analyses of TAN, PO_4^{3-}-P, NO_3^--N, NO_2^--N, VFAs, and sCOD using a 20 mL syringe. At the same time, pH and temperature were monitored using a portable meter (Hach HQ40d, Hach Lange, Germany). TCOD, TP, and TKN were measured in the reactors at the start of the anoxic/anaerobic degradation period (day 0).

2.6 Data treatment

2.6.1 The degree of solubilization and degree of fermentation

The organic waste degradability was quantified as the degree of solubilization expressed as the dissolution of COD (Equation1 (Eq 1)), while the capacity of the organic waste to produce VFAs was quantified and expressed as the degree of fermentation (Equation 2 (Eq.2)).

$$\textbf{Degree of solubilization} = \frac{\text{sCOD}}{\text{TCOD}} \quad \text{(Eq.1)}$$

$$\textbf{Degree of fermentation} = \frac{\text{VFA\_COD}}{\text{sCOD}} \quad \text{(Eq 2.)}$$

2.6.2 The System mass balance analysis

The SSF and the denitrification reactor performance were evaluated under a mass balance approach, where: accumulation = input – output + generation. The SSF was defined as a control volume were IN values correspond to the masses discharged from the backwash of the drum filter, while OUT values correspond to the masses discharged into the denitrification reactor (0.48 m^3/d) (Equation 3 (Eq. 3)) (Figure 1). Similarly, the denitrification reactor was defined as the control volume, where in this case IN corresponds to the masses entering the reactor, namely the water pumped from the large biofilter (6, 18 and 54 m^3/d) and the SSF. OUT values correspond to masses leaving the denitrification reactor into the wetland (Equation 4 (Eq. 4)) (Figure 2).

$$V * \frac{dC_{SSF}}{dt} = Q_o * C_{SSF,o} - Q * C_{SFF,effluent} + r_{SSF} * V \quad \text{(Eq. 3)}$$

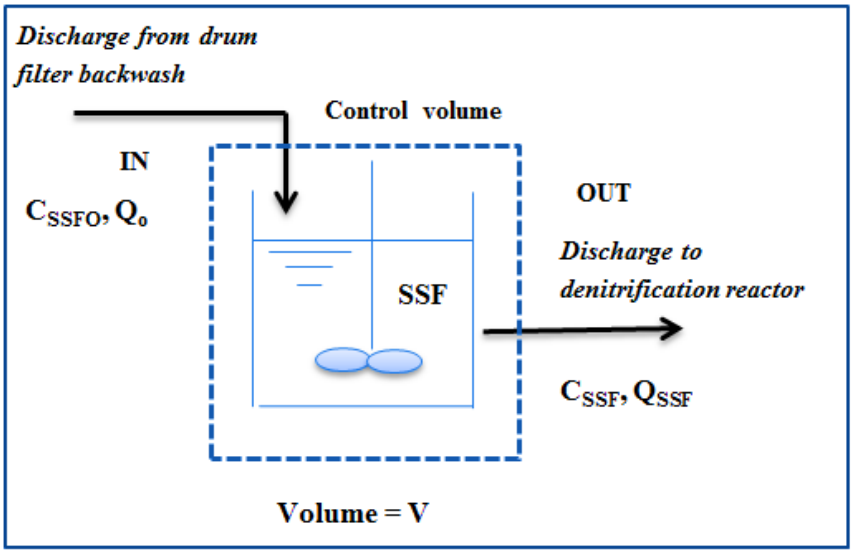


Fig. 2: Mass balance on SSF

Where:

dC_{SSF}/dt = rate of change of reactant concentration within the control volume (g/m^3*d)

V = reactor volume (control volume) (m^3)

Q_0, Q_{SSF} = volumetric flow rates (m^3/d)

C_{SSFo}, C_{SSF} = concentration of SSF in the influent and effluent (g/m^3)

r_{SSF} = volumetric reaction rate (generation or consumption rate) (-1/d)*(g/m^3)

$$V * \frac{dC_{Deni}}{dt} = (Q_{SSF} * C_{SSF} + Q_{Bio} * C_{Bio}) - Q_{Deni} * C_{Deni,effluent} + r_{Deni} * V \quad \text{(Eq. 4)}$$

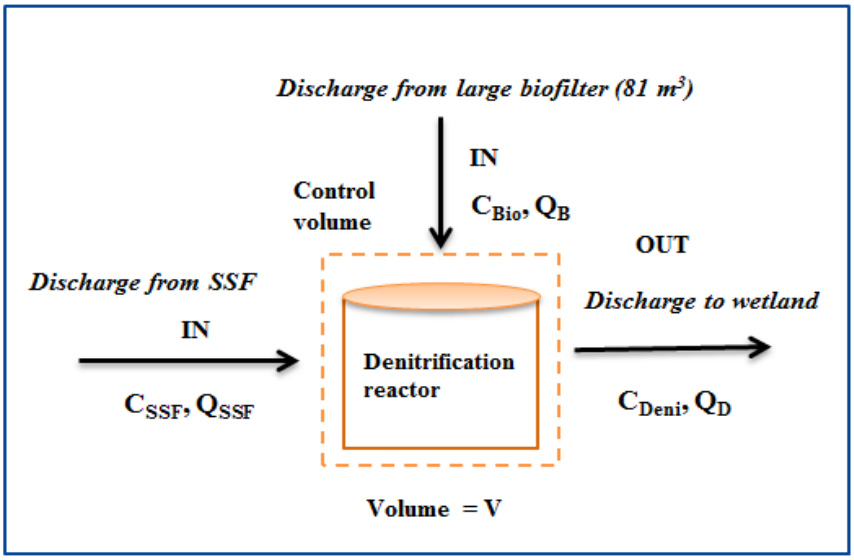


Fig. 3. Mass balance on the denitrification reactor

Where:
dC_{deni}/dt = rate of change of reactant concentration within the control volume (g/m^3*d)
V = reactor volume (control volume)
Q_B, Q_{SSF}, Q_{Deni} = volumetric flow rates (large biofilter, side stream fermenter and denitrification reactor) (m^3/d)
C_{SSF}, C_B, C_{Deni} = concentration of side stream fermenter and biofilter in the influent and the denitrification reactor at effluent (g/m^3)
r_{Deni} = volumetric reaction rate (generation or consumption rate) ($-1/d)*(g/m^3$)

2.7 Analytical methods

Samples for anions (NOx-N, PO_4^{3-}P), sCOD and VFAs were centrifuged at 4500 rpm for 15 min at 0ºC immediately after they were obtained, and filtered through 0.2 µm syringe filters (Filtropour S, SARSTEDT, Germany). The filtered samples for VFAs, sCOD and raw samples for TCOD, TP and TKN were subsequently preserved by adding 1% v/v sulfuric acid (4 Mol/L H2SO4, Merck Millipore, Germany), and maintained at 4ºC until further analysis. Anions (NOx-N, PO_4^{3-}P) were analyzed using a 930 Compact IC Flex 1 with a Metrosep A Supp 7 -250/4.0 column type, coupled with a 887 Professional UV/VIS detector (Metrohm, Sweden), 0.1 M H_2SO_4 was used as suppressor and 3.6 mN Na_2CO_3 was used as eluent. VFAs were analyzed using an 881 Compact IC pro with a Metrosep organic acids – 250/7.8 column type (Methrom, Sweden), 0.1 M LiCl was used a suppressor and 0.5 mM H_2SO_4 as eluent. Determination of TCOD was performed using digestion vials (LCK 514, Hach Lange, Germany), sCOD was analyzed using digestion vials (LCK 314, Hach Lange, Germany) and TKN was determined by digesting and distilling the samples according to ISO 5983-2 (2005). pH, DO and temperature were measured using a portable meter (Hach HQ40d, Hach Lange, Germany).

3. RESULTS

3.1 Characteristics and degradability of the obtained organic waste

The organic waste characterization for C, N and P forms obtained from the backwash of the drum filter from the different sources (hatchery, small biofilter, large biofilter and sludge cones) are presented in Table 2. According to the results obtained from the 7 days degradability laboratory trials, the degree of solubilization ranged between 1.4-10.6% (14.0 – 106 mg sCOD/g TCOD) (Figure 4a) while the degree of

fermentation ranged between 12-22% (0.12 – 0.22 mg VFA_COD/mg sCOD) (Figure 4b). The hatchery and the small biofilter showed increased values for these two parameters between the organic waste sources, reaching VFAs concentrations of 18.7 ± 5.9 mg VFA_COD at day 2 and 17.4±2.1 mg VFA_COD at day 3, respectively. The smallest potential for degradability was found for the large biofilter reaching a degree of fermentation of 0.12 ± 0.01 at day 1 with a production of 5.16 mg VFA_COD/mg sCOD at that same period of time.

Table 2. C, N and P composition of the different organic waste sources used in the SSF (mean ± SD, n=3).

Device	Carbon			Nitrogen				Phosphorous	
	TCOD g/L	sCOD mg/L	VFA mg/L	TN mg/L	NO_3^-N mg/l	NO_2^-N mg/L	NH_4^+-N mg/L	TP mg/L	PO_4^{3-}-P mg/L
Hatchery	0.8±0.2	48.3±27	2.3±0.5	48.1±13.2	4.7±0.4	0.6±0.0	1.6±0.6	19.4±4.4	0.5±0.0
Sludge cones	1.6±0.4	24.5±6.1	2.2±1.3	80.4±17.4	5.0±0.0	0.1±0.1	1.3±0.8	61.4±11.1	0.2±0.1
Small Biofilter	3.3±0.7	34.6±19.8	1.6±0.8	170.8±33.6	4.7±0.5	0.6±0.4	1.0±0.3	96.4±21.5	2.7±2.9
Large Biofilter	2.3±0.4	15.7±2.5	1.0±0.4	128.3±22.7	5.1±0.4	0.3±0.0	0.6±0.4	78.5±12.6	0.3±0.1

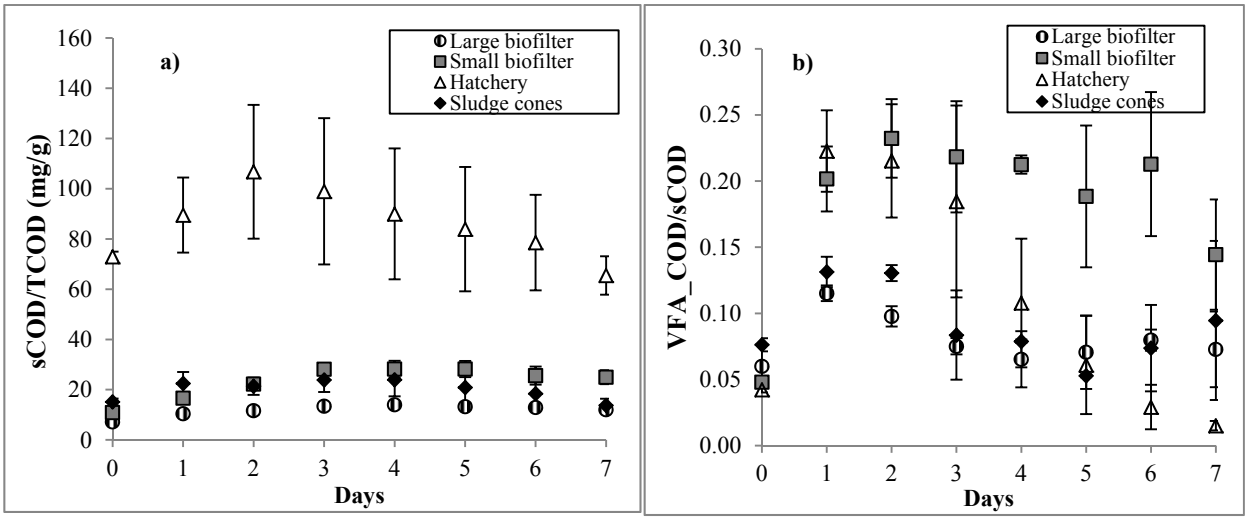


Fig. 4. a) Cumulative degree of dissolution (sCOD/TCOD) found in the different organic waste types during 7 days of anoxic/anaerobic degradation performed in laboratory conditions (mean ± SD, n=3). **b)** Cumulative degree of fermentation (VFA_sCOD/sCOD) from different organic waste types during 7 days of anoxic/anaerobic degradation performed in laboratory conditions (mean ± SD, n=3).

3.2 Performance of SSF

The different nutrients and organic matter forms expressed as concentrations in the discharge of the SSF illustrate the process stability during the experimental period (42 days) (Table 3). Total COD values averaged 2.9 ± 1.4 g/L, showing variability due to the properties of the sludge (high settleability). The dissolved organic matter concentrations were more stable as sCDO values ranged 68.6 ± 19.9 mg/L and VFAs averaged 23.5 ± 4.2 mg/L (Table 3). NO_3^--N and NO_2^--N discharged from the SSF were constantly below the detection limits were as NH_4^+ and PO_4^{3-}-P were produced at constant concentrations of 13.1±4.5 mg NH_4^+-N/L and 3.4±0.5 mg PO_4^{3-}P/L. From the measured VFAs, acetate was the main compound found with an average value of 22.9 ± 3.9 mg/L corresponding to 97.4% of total VFAs measured. The degree of solubilization was 2.3% (0.23 mg sCOD/mg TCOD) while the obtained degree of fermentation was 34%

(0.34 mg VFA_COD/ mg sCOD). The pH values measured inside the reactor during the trial were 7.0 ± 0.1 with a temperature of 12.9 ± 0.9°C and a dissolved oxygen concentrations <0.2 mg/L.

3.3 Denitrification reactor performance under different evaluated flows (6, 18 and 54 m^3/d)

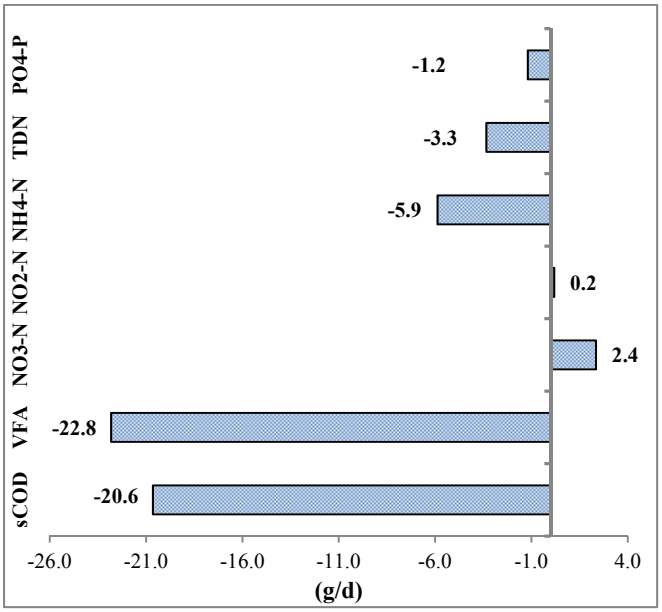
The performance of the denitrification reactor depended on the different operational flows as seen on the NO_x concentrations found in the effluent of the reactor (Table 3). The NO_3-N concentration entering the reactor was relatively constant (5.4±0.4 mg-N/L), while the effluent concentration varied according to the different evaluated operational flows 6, 18 and 54 m^3/d (1.3±0.8, 3.8±0.8 and 5.0±0.2 mg NO_3^--N/L, respectively). The concentration of oxygen entering the reactor showed to be relatively constant (4.14 ±1.8 mg/L), similarly as the values for the effluent in the first two flows (<0.15 ± mg/L). In the case of the third flow (54 m^3/d) oxygen effluent values varied between 0.5-1.0 mg/L. The NH_4^+- N concentrations in the effluent of the denitrification decreased as the operational flows of the denitrification reactor increased, similarly as with the concentration values for PO_4^{3-}- P, sCOD and TP (Table 3). VFA values found in the effluent maintained constant independently of the operational flow as well as TN. TCOD values found in the effluent at the initial flow of 6 m^3/d were double and with more variability than found for 18 and 54 m^3/d. The pH values of the water entering the denitrification reactor were 7.5±0.1 at a temperature of 12.5±1.0, while at the discharge of the denitrification reactor the measured values for pH were 7.3±0.1 at a temperature of 13.5°C ±1.0.

Table 3. Concentration values found in the discharge of the SSF (IN C_{SSF}) and IN/OUT concentration values from the denitrification reactor at different operational flows (mean ± SD, n=7).

Parameter	Flows				Flows		
	0.48 m^3/d	6.0 m^3/d	18.0 m^3/d	54.0 m^3/d	6.48 m^3/d	18.48 m^3/d	54.48 m^3/d
	(OUT C_{SSF})	(IN $C_{denitrification}$)			(OUT $C_{denitrification}$)		
NO_3^--N	0.0±0.0	5.0±0.2	5.5±0.4	5.8±0.3	1.3±0.8	3.8±0.8	5.0±0.2
NO_2^--N	0.0±0.1	0.1±0.0	0.2±0.0	0.2±0.0	0.2±0.1	0.2±0.1	0.2±0.0
NH_4^+- N (mg/L)	13.1±4.5	1.5±1.8	0.8±0.3	0.8±0.4	4.0±1.3	1.6±0.5	0.9±0.4
PO_4^{3-}-P (mg/L)	3.4±0.5	0.2±0.0	0.2±0.0	0.3±0.0	1.3±0.5	0.5±0.1	0.3±0.0
sCOD (mg/L)	68.6±19.9	8.0±1.2	8.4±5.6	7.7±0.9	13.1±7.4	8.4±5.6	7.6±1.7
VFA_COD (mg/L)	13.1±4.5	0.5±0.5	0.6±0.5	0.8±0.4	0.9±0.2	0.7±0.3	0.9±0.6
TCOD (mg/L)	2943±1427	10±2.4	11.8±4.0	13.5±3.0	20.2±10.0	10.3±2.0	11.3±3.5
TP (mg/L)	104.9±52.3	0.3±0.1	0.7±0.7	0.4±0.0	2.1±0.9	1.0±0.2	0.5±0.08
TN (mg/L)	139.5±84.9	7.1±0.3	8.3±2.7	7.3±0.3	6.3±0.6	6.2±0.1	6.4±0.3

3.4 Mass balance on the SSF

Under the mass balance analysis (Figure 5) the SSF managed to increase in 70% the mass of sCOD and 14 times fold the VFA mass entering the reactor. This represents a constant mass of 20.6 g/d of sCOD and 22.8 g/d of VFA discharged to the denitrification reactor. NO_3^--N and NO_2^--N entering the SSF were consumed at amounts of 2.4 and 0.2 g/d. Dissolution of 5.9 g/d NH_4^+-N was found in the discharged water, corresponding to an increment of 14 times fold the mass of NH_4^+-N entering the reactor. In a similar way 1.1 g/d of PO_4^{3-}-P were discharged from the reactor, corresponding to and increment of 2.6 times folds the mass of PO_4^{3-}-P entering the reactor. The consumption of NOx in the reactor and the production of NH_4^+-N resulted in a net production of 3.3 g N/d accounted as total dissolved nitrogen (TDN) (Figure 5).



253

254 **Fig. 5:** Mass balance results on the SSF, positive (+) values represent consumption while negative (-) values represent accumulation.

255 ### 3.5 Mass balance on the denitrification reactor

256 The mass balance analyisis on the denitrification reactor showed that the higher removal of NO_3^--N
257 (42.4 g/d) was found at the higher flow (54 m^3/d), which corresponded to 13% of the NO_3^--N that entered the
258 reactor (Figure 6). At the lower flows (6 and 18 m^3/d) 25.3 and 24.7 g/d of NO_3^--N were removed
259 respectively, which corresponded to 75% and 26% of the NO_3^--N mass that entered the reactor. A 13.2%
260 (52.7 g/d) reduction of TN was found at a flow of 54 m^3/d where 16% (40.6 g/d) and 26% (7.8 g/d) reduction
261 was found respectively for the 6 and 18 m^3/d flows. The NH_4^+- N mases at the effluent of the denitrification
262 reactor varied according to the operational flow. A 63% mass production (9.9 g NH_4^+- N /d) and 40%
263 production (8.6 g NH_4^+- N /d) were respectively registered at 6 and 18 m^3/d flows, while only 4% (2 g/d g
264 NH_4^+- N /d) was produced at a flow of 54 m^3/d. The removal of NO_3^--N and production of NH_4^+- N balanced
265 the final masses of TDN removed as at a flow of 54 m^3/d practically three times mass removal was achieved
266 compared to the two evaluated flows (Figure 6). The mass of oxygen consumed increased as the operational
267 flows increased, consuming 23.8 gO_2/d at 6 m^3/d (96% consumption), 77.7 g O_2/d at 18 m^3/d (98%
268 consumption) and 181.7 g O_2/d at 54 m^3/d or 86% of total mass of oxygen that entered the reactor.

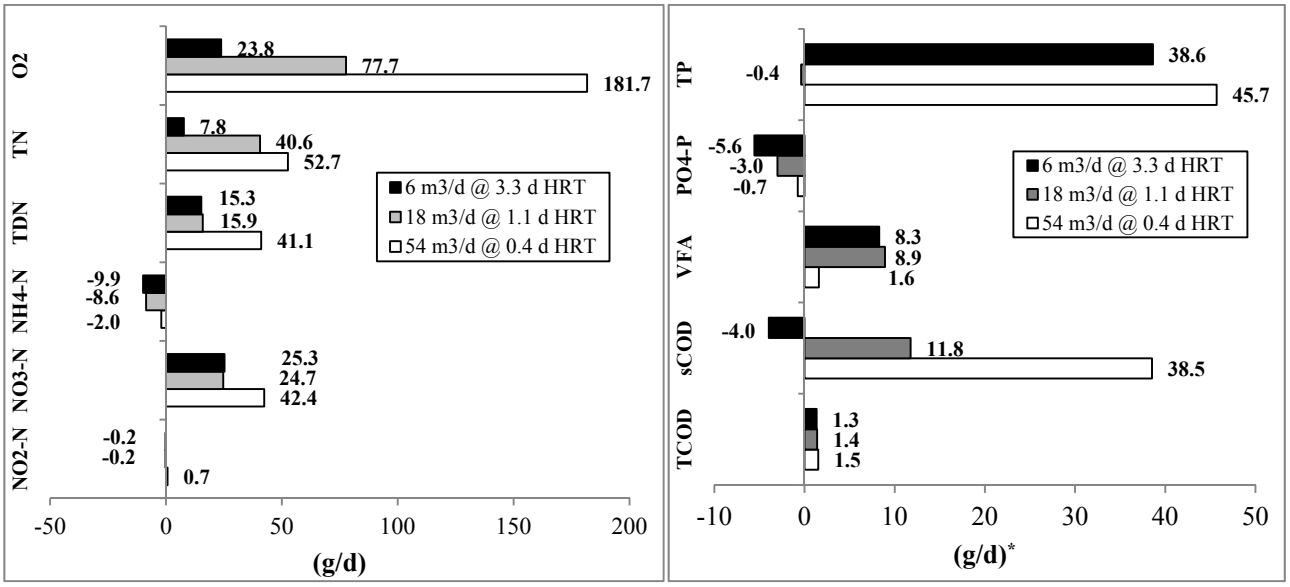


Fig. 6. Mass balance on the denitrification reactor at three different evaluated flows (6, 18 and 54 m^3/d). Positive (+) values represent consumption while negative (-) values represent accumulation. *TCOD values are expressed in Kg/d.

The TCOD mass removed varied between 91% (1.3 Kg TCOD/d) to 71.2% (1.5 Kg TCOD/d) as the operational flows of the denitrification reactor increased from 6 to 54 m^3/d (Figure 6). The higher mass of sCOD removed was found at a flow of 54 m^3/d with a consumption of 38.5 g/d or 9% of the mass that entered the denitrification reactor. At a flow of 18 m^3/d the sCOD consumption corresponded to 11.8 g/d or 7% of the mass that entered the reactor, while an accumulation of sCOD (4 g/d) or 5% of the mass of sCOD that entered the denitrification reactor was found at 6 m^3/d. VFA consumption showed to be higher at 6 (8.3 gVFA/d or 58% consumption) and 18 m^3/d (8.9 gVFA/d or 41% consumption) as compared to 54 m^3/d (1.6 gVFA/d or 3% consumption). PO_4^{3-}-P mass production decreased as the flow increased. In this sense, 5.6 g PO_4^{3-}-P/d were produced at a flow of 6 m^3/d (195% production), whereas at a flow of 18 m^3/d, the production was reduced to 3.0 g PO_4^{3-}-P/d (53% production). At 54 m^3/d a production of 0.7 g PO_4^{3-}-P/d or a 4% increment of the mass of PO_4^{3-}-P entering the reactor was found. TP was reduced at the lower and higher flows (6 and 54 m^3/d) with 28.4 g TP/d and 35.5 g TP/d corresponding to 68% and 56% consumption, respectively. At a flow of 18 m^3/d a TP production of 3% was found which corresponded to 0.4 g TP/d (Figure 6).

4. Discussion

4.1 Side stream fermenter (SSF)

In general terms the side stream SSF showed to deliver a constant amount of readily carbon sources. However, the low quality of the organic waste collected was reflected in the low degree of solubilization (sCOD/TCOD), as approximate only 2% of the available TCOD was converted to sCOD as found in the laboratory trials and the farm trial. In this respect, the obtained values are similar as described by Ucizik and Henze (2008) for degradability of organic matter obtained from an activated sludge system in a wastewater treatment plant. Indicating the highly degraded state of the organic waste presumably composed of bacterial mass. The low organic waste quality relates mainly to the farm configuration, as the organic waste have been submitted to saturated oxygen water conditions and long retention times in the farm water circuit before its collection. Because of this the easily degradable fraction of the organic matter has already being lost or consumed by bacteria before entering the SSF. Additionally, the collection method (drum filter) majorly

collected the particulate fraction while the soluble fraction was lost as passed through the drum filter mesh. Comparing the degree of solubilization obtained in this trial with other studies from aquaculture organic waste, the obtained values were 10 to 15 times lower. Letelier-Gordo et al. (2015) reported degree of dissolution values between 230-300 mg sCOD/g TCOD from organic waste collected in settling cones. Similar values were reported by Conroy and Couturier (2009) (400 mg SCOD/ g TVS) from organic waste collected in swirl separators and Suhr et al. (2012) (200-300 mg sCOD/g TCOD) from different organic waste (sludge cones and drum filter backwash). In this sense is evident the importance on how the organic waste is collected and the time the organic waste has spent in the RAS treatment circuit as for obtaining its full potential for using it as internal carbon source.

In terms of the degree of fermentation (VFAs_COD/sCOD) the data obtained from the laboratory trials showed that VFA accumulation reached for almost all organic waste types a maximum concentration between day 2 and 3 before being consumed. Thus the HRT of 7 days set for the SSF can be consider as non-optimal according to the laboratory trials as consumption happens from day 3 onwards. In this respect, we have to highlight that 7 days were chosen for experimental purposes in order to standardize the collection of the different organic waste sources. Unfortunately, not much information is available in terms of the degree of fermentation for aquaculture organic waste where different days have been reported for achieving maximum production yields. Suhr et al. (2013) reported maximal VFA yields at 5 days of anaerobic degradation of the organic waste using an anaerobic fed batch reactor, Conroy and Couturier (2009) reported maximal VFA yield at 10 days and Letelier-Gordo et al. (2015) found a maximal VFA yields at 4 days, the two last authors used an anaerobic batch reactor for the evaluation. In a study concerning methanogenesis from aquaculture organic waste, Mirsoyan and Gross (2013), found COD removals over 98% and concomitant methane production in an upflow anaerobic sludge blanket (UASB) reactor operated at 6 and 8 days of HRT. This last study reflects the influence methanogens can have in the HRT required for organic waste degradation as VFAs are intermediate products for methanogenesis. In the present study, it called the attention that the VFAs yields obtained in the SSF were higher (34%; $p<0.05$) as compared to the values obtained in the laboratory (10-18%). Most probably methanogenic or sulfate reducing bacterial populations stablished in the SSF creating more stable condition for acetogenic bacteria to degrade short chain fatty acids (i.e. propionate and butyrate) into acetate. This last condition reflected as majorly acetate and in some extend formate (2.6% of total VFAs) was measured in the SSF, while in the case of the laboratory trials butyric, valeric, propionic, acetic and formic acids accumulated in the batch reactors. In this sense, methanogenic and sulphate reducing bacteria could have utilized molecular hydrogen, creating a syntrophic association with the acetogenic bacteria (interspecies hydrogen transfer) allowing acetogenic bacteria to degrade short chain fatty acids to acetate under exergonic conditions (energetically favored) (Henze et al., 2008; Muyzer and Stams, 2008). Additionally, low concentrations of sulfate were found in the SSF (1.3 mg SO_4-S/L) (values not reported) and more stable pH values (7.0 ± 0.1) as compared to the laboratory trails (values averaged 7.4 ± 0.3 at day 0 and decreased to an averaged 6.6 ±0.1 until day 7) reinforcing the establishment of a well-developed anaerobic digestion process in the SSF. In a general perspective, defining an optimal day in which the maximal VFA yield can be achieved and thus the definition of the required HRT for the SSF is not clear and it seems it depends in the source of organic waste and the associated establishment of methanogenic or sulphate-reducing bacterial community. Laboratory trials characterizing the organic waste degradability can be an inexpensive method to define the optimal HRT for the SSF and thus avoiding a miss use of the internal carbon sources available and a suboptimal reactor volume with the corresponding associated costs.

A possible drawback of using the organic waste as internal carbon source is the dissolution of NH_4^+ and PO_4^{3-} (Conroy and Couturier, 2009; Letelier-Gordo et al., 2015). In the present experiment the SSF

constantly produced 14 times more mass of NH_4^+ and 2.6 times more PO_4^{3-} compared to the initial masses found at the organic waste at day 0. The discharged masses and concentrations of NH_4^+ and PO_4^{3-} varied according to the operational flow of the denitrification reactor were the lowest discharged concentrations (0.3 mg PO_4^{3-}P/L and 0.9 mg /L NH_4^+- N) were found at 54 m^3/d. Apparently PO_4^{3-} concentrations in the discharge does not seem to be a problem, although NH_4^+- N concentrations are relatively high for discharge values. The involved dynamics for these two parameters at different flows the denitrification reactor was operated will be explained further.

4.2 Denitrification reactor

Contrary to our expectations the performance of the denitrification reactor in terms NO_3^--N removal did not behave accordingly to the different flows applied. In the first evaluated flow (6 m^3/d) practically all oxygen that entered the reactor was consumed 96% (23.8 gO_2/d) and 75% of the NO_3^--N (25.3 g NO_3-N/d), having an average effluent of 1.3±0.8 mg NO_3^--N/L. Additionally the mass balance for sCOD showed an accumulation/production of 4 gsCOD/d (5%) and in the case of TCOD a reduction in mass was found but with a high variability of concentration in the effluent (20.2±10 gTCOD/L). Under these facts we can state that the low flow entering the reactor (HRT 3.3 d) resulted in a system operated under limited substrate (NO_3^-) conditions with organic matter being accumulated and degraded inside as shown in the TCOD mass removed, and production of sCOD, NH_4^+ and PO_4^{3-} .The second evaluated flow (18 m^3/d) supposed to show the higher removal of NO_3^-, as 98% of the oxygen was consumed (77.7 gO_2/d) and anoxic conditions and more substrate (NO_3^-) were available to remove. Unfortunately, the results did not reflect these conditions as the amount of NO_3^- removed was similar as obtained in the first evaluated flow (18 m^3/d). Eventually carbon limitation could have been a reasonable explanation for this situation. However, at the third evaluated flow (54 m^3/d) a higher mass of oxygen was reduced 181.7 (gO_2/d) with anoxic/aerobic conditions found in the effluent at the limit values (0.5-1.0 mg/L) for denitrification (Henze et al., 2008) and double amount of NO_3 was removed compared to the two initial flows. The underlying reason could be an unmixed condition inside the reactor at the intermediate flow (18 m^3/d) creating channeling through the media which was disrupted when a higher flow (54 m^3/d) was applied, thus the contact between the bacteria and the substrates (organic matter and NO_3^-) was improved.

Interestingly, the mass of PO_4^{3-} discharged from the denitrification reactor decreased as the operational flow increased, varying from a discharge of 5.6 g/d at an operational flow of 6 m^3/d (1.9 times fold production) to 0.7 g/d (4% production) at 54 m^3/d. Unfortunately studies using internal carbon sources for denitrification have not focused in the involved dynamics related to PO_4^{3-} or TP, majorly reporting organic matter and nitrate dynamics. In a study developed by Barak and van Rijn (2000) it was reported denitrifying bacteria are capable of uptaking phosphate in excess in the presence of nitrate, finding that the content of organic matter in a fluidized bed reactor was high as 11.8%. In waste water treatment, phosphorous accumulating organisms (PAOs) can also uptake up to 38% of P per amount of bacteria in the presence of VFAs when anaerobic and aerobic conditions are alternated (Henze et al., 2008). In the present study samples characterizing the bacteria present in the denitrification reactor media was not performed for P content although the organic particulate fraction obtained in the effluent of the reactor (assumed as bacteria mass) showed to have between 5-25% of P content exceeding normal P contents of 2% as reported by (Metcalf and Eddy, 2004). Considering that the SSF produced VFAs under anaerobic conditions and posteriorly inside the denitrification reactor anoxic and aerobic conditions were found, the removal of PO_4^{3-} produced from the degradation of organic matter inside the denitrification reactor (reflected in the mass balance) could have been uptaken by the activity of one of the above mentioned bacterial processes.

According to the mass balance NH_4^+ was produced inside the denitrification reactor as presumably organic matter was degraded. The mass of NH_4^+ produced was similar for the two first flows (6 and 18 m^3/d) while for the third flow the mass of NH_4^+ was reduced in 20% as compared to the two first flows. The unstable anoxic and aerobic conditions found in the third evaluated flow (54 m^3/d) could have promoted nitrification explaining the reduction in the NH_4^+ mases and concentration found in the effluent (0.9±0.4 mgNH_4^+-N/L). Even though 0.9±0.4 mg NH_4^+-N/L is considered as a high value for NH_4^+ discharge a further step on reducing these parameters could be the recycling of a fraction of the effluent back into the inlet of the denitrification reactor. In this sense if we use the NH_4^+ concentration found in the effluent at the most optimal flow (0.9±0.4 mg NH_4^+-N/L at 54 m^3/d) and we assume that 20% of the operational flow of the denitrification reactor will be recirculated, 9.72 g/d of NH_4^+ could eventually be removed by nitrification. Thus this will result in a consumption of 40.6 g/d of O_2 (4.18 g O_2/g N (Timmons et al., 2008)) improving simultaneously the availability of carbon sources for the denitrification reactor and simultaneously reducing the associated discharge values for NH_4^+.

4.3 Evaluation of implementing a SSF reactor on Danish rainbow trout brood stock farm

Denitrification capacity using endogenous carbon sources in aquaculture have shown some variability, Suhr et al. (2012) reported a denitrification capacity of 125 g NO_3^--N/m^3 reactor/d or 60 g NO_3^--N/kg TVS/d at a HRT of 98 min in a commercial rainbow trout farm using 5.5 m^3 upflow reactors with an inlet oxygen content of 4 mg/L. Klas et al. (2006) found an average nitrate removal rate of 120-150 gNO_3^--N/m^3 reactor/d finding interference with oxygen in the removal rates, although values are no presented. Tsukuda et al. (2014) reported removal rates of 402 g NO_3^--N/m^3 biofilter/d using endogenous carbon sources produced form the backwash of a drum filter and settling cones from rainbow trout (*Oncorhynchus mykkis*) and Atlantic salmon (*Salmo salar*) RAS. The inlet oxygen concentrations in the denitrification reactor (fluidized sand biofilter) were less than 0.37 mg O_2/L. It is evident how the different removal rates reported depended on system configuration, and operational conditions as masses of carbon and oxygen entering the system as well as temperature. According to the data obtained for the most optimal flow operating the denitrification reactor (54 m^3/d), 1 m^3 of the enhanced sludge in the SSF was able to remove 93.2 g of NO_3^--N plus 379 g of oxygen. In this case study, the trout farm discharged 2160 m^3/d of water with an associated mass of 11.6 Kg NO_3^--N/d and 8.8 Kg O_2/d. To comply with environmental regulations, the farm needs to reduce the actual discharge of TN by 22%, meaning that 2.5 Kg NO_3^--N/d should be removed. To do so, 27 m^3 of organic waste should be treated by the SSF each day, removing in addition 10.3 Kg O_2/d from the effluent to achieve the required anoxic conditions. This is far beyond the amounts of organic waste that the farm produces each day, and use of external carbon sources would probably be required to comply with the environmental regulations. Further improvements could, however, be made to the system to improve the process performance, and in this way reduce the cost of purchasing external carbon sources. The improvements may include:

a) ***Reduce the oxygen concentration*** **entering the denitrification reactor**.

If oxygen is present in the water (> 1 mg/L), bacteria will always use oxygen over nitrate for metabolic processes (energetically favored) this will influence in the final carbon budget to perform denitrification using endogenous carbon sources. Thus stoichiometrically speaking 0.7 Kg of organic waste expressed as COD is required to remove 1 Kg of O_2 while 2.86 Kg or organic matter expressed as COD are required to reduce 1 Kg of N, meaning that per every Kg of O_2 removed in the affluent a 24% reduction in the denitrification capacity using internal carbon sources is estimated.

b) ***Apply a flow loop between the effluent and the affluent of the denitrification reactor.***

According to the results obtained, not all sCOD and VFAs were consumed in the denitrification reactor, which was probably due to fluid dynamic problems inside the reactor and associated low concentrations affecting the half saturation constants for bacteria to use the available carbon substrate (e.g.10-20 gCOD/m^3 for denitrifiers (Henze et al., 2002)). Therefore, recycling the effluent water into the influent of the denitrification reactor would, in theory, increase the usage of the discharged compounds by reducing oxygen masses, and eventually increase the concentration of the substrate, thus leaving more carbon from the SSF to reduce nitrate. Additionally, this configuration would help to reduce the concentrations of NH_4-N discharged from the denitrification reactor. If 20% of the optimal flow founded in this trial (54 m^3/d) is recycled back to the influent of the denitrification reactor, 9.72 g/d of NH_4^+ could eventually be removed by nitrification. This will result in a consumption of 44 g/d of O_2 improving simultaneously the capacity of the denitrification reactor (30.4 g of COD available for denitrification) and the discharge values for NH_4^+.

c) Improve the quality of the recovered carbon.

Removing and collecting the organic waste from the raceways in an efficient way, and thus avoiding the constant degradation under aerobic water conditions, will increase the amount of easily degradable organic waste and give higher sCOD/TCOD yields. In the present case, 2% of the collected organic matter was transformed into sCOD, whereas usually obtained values range between 20-30% of sCOD from the TCOD. Moreover, a separation of the different organic waste types may be considered. In the present evaluation, all the organic waste derived from the backwash of the drum filter, and the dissolved fractions of the organic waste was therefore lost. This applied specifically to the organic waste coming from the hatchery and sludge cones, containing a higher fraction of dissolved organic matter and with a better degradability as compared to that coming from biofilter backwash. In this sense, a recommendation would be to discharge all the water from the hatchery or sludge cones directly into the SSF, while applying the drum filter only when treating the organic waste coming from the biofilter backwash.

d) Improve the internal fluid dynamics in the denitrification reactor

The use of media to allow bacterial attachment is a good solution for decoupling the HRT from the bacteria biomass, thus avoiding bacterial washout. However, if the media is not correctly mixed, channeling of the flows inside the reactor may occur, especially at low flows. This results in a suboptimal usage of the media and an erratic behavior of the reactor. The performance of the denitrification reactor turned out to be flow dependent with the mixing conditions affecting the removal capacity. Improvement on the mixing mechanism of the media, or eventually dimensioning the system to operate as plug-flow, will increase the contact time between the substrate and the bacteria especially under low carbon flows.

5. Conclusions

Using a SSF for producing readily available carbon sources showed to enhance and deliver a continuous mass of carbon sources for the denitrification reactor while decoupling the associated HRT between the solubilization of the organic waste and the denitrification operational flow. Attention must be focus on the discharged concentrations of NH_4^+ and PO_4^{3-}.

The amount and degradability capacity of carbon sources recovered from the cleaning operations of the treatment units limited the denitrification capacity of the system due to the highly reduced estate of the carbon sources and the high oxygen levels in the treated water.

To achieve the environmental discharged regulations the required amount of organic waste for the denitrification system is beyond the amounts of organic waste collected in the actual configuration of the farm. Thus the use of external carbon sources would probably be required to comply with the environmental regulations. Although, the application of this technology will help to reduce the amount of external carbon sources required at first to transform the effluent anaerobic conditions to anoxic conditions thus achieve denitrification.

Acknowledgments

This research was funded by The Ministry of Food, Agriculture and Fisheries of Denmark and by the European Union through The European Fisheries Fund (EFF). The technical skills and invaluable assistance of Mathis von Ahnen, Melisa Lyng, Brian Møller and Ulla Sproegel are highly appreciated.

References

Conroy, J., Couturier, M., 2010. Dissolution of minerals during hydrolysis of fish waste solids. Aquaculture 298 (3), 220–225.

Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K., Pedersen, P.B., 2013. Farming different species in RAS in Nordic countries: Current status and future perspectives. Aquacultural Engineering 53, 2-13.

Danish Ministry of Environment, 2012. Bekendtgørelse om miljøgodkendelse og samtidig sagsbehandling af ferskvandsdambrug (Executive order on the environmental approval and handling of freshwater aquaculture systems). Lovtidende A nr. 130. Miljøstyrelsen, Miljøministeriet, Denmark (in Danish).

Jewell, W.J., Cummings, R.J., 1990. Expanded bed treatment of complete recycle aquaculture systems. Water Science and Technology 22, 443-450.

Jokumsen, A Svendsen, L., 2010. Farming of Freshwater Rainbow Trout in Denmark. DTU Aqua, National Institute of Aquatic Resources. Report no 219-2010, DK. 47 pp.

Diaz, V., Ibanez, R., Gomez, P., Urtiaga, A.M., Ortiz, I., 2012. Kinetics of nitrogen compounds in a commercial marine recirculating aquaculture system. Aquacultural Engineering 50, 20-27.

Hamlin, H.J., Michaels, J.T., Beaulaton, C.M., Graham, W.F., Dutt, W., Steinbach, P., Losordo, T.M., Schrader, K.K., Main, K.L., 2008. Comparing denitrification rates and carbon sources in commercial scale upflow denitrification filters in aquaculture. Aquacultural Engineering 38, 79-92.

Henze, M., Harremoës, P., Jansen, J.l.C., Arvin, E., 2002. Wastewater treatment. Biological and chemical process. Springer 3rd edition. 430 pp.

Henze, M., van Loosdrecht, M., Ekama, G. A., Brjanovic, D., 2008. Biological Wastewater Treatment: principles, modelling and design. IWA Publishing 2008, 511 pp.

ISO 5983, 2005. Animal Feeding Stuffs – Determination of nitrogen content and calculation of crude protein content – Part 2: Block digestion/steam distillation method. International Organization for Standardization, Geneva, Switzerland, 14 pp.

Klas, S., Mozes, N., Lahav, O., 2006. A conceptual, Stoichiometry-based model for single-sludge denitrification in recirculating aquaculture systems. Aquaculture 259, 328 – 341.

Letelier-Gordo, C.O. Dalsgaard, J., Suhr, K., Ekmann, K.S., Pedersen, P.B., 2015. Reducing the dietary protein:energy (P:E) ratio changes solubilization and fermentation of rainbow trout (Oncorhynchus mykiss) faeces. Aquacultural Engineering. 66, 22-29.

Martins, C.I.M., Eding, E.H., Verdegem, M.C.J., HeinSSFoek, L.T.N., Schneider, O., Blancheton, J, E., Roque d'Orbcasteld, J., Verreth, A.J., 2010. New developments in recirculating aquaculture systems in Europe: a perspective on environmental sustainability. Aquacultural Engineering 43, 83–93.

Metcalf, E., 2004. Wastewater Engineering, Treatment and Reuse, 4th ed. McGraw Hill, 1819 pp..

Muyzer, G., Stams, A. J. M., 2008. The ecology and biotechnology of sulphate-reducing bacteria. Nature reviews microbiology 6, 441-454.

Otte, G., Rosenthal, H., 1979. Management of a closed brackish water system for high density fish culture by biological and chemical water treatment. Aquaculture 18, 169-181.

Suhr, K.I., Pedersen, P.B., Arvin, E., 2012. End-of-pipe denitrification using RAS effluent waste streams: Effect of C/N-ratio and hydraulic retention time. Aquacultural Engineering 53, 57-64.

Suhr, K.I., Pedersen, L.-F., Nielsen, J.L., 2014. End-of-pipe single-sludge denitrification in pilot-scale recirculating aquaculture systems. Aquacultural engineering 62, 28-35.

Suzuki, Y., Maruyama, T., Numate, H., Sato, H., Asakawa, M., 2003. Performance of a closed recirculating system with foam separation, nitrification and denitrification units for intensive culture of eel: towards zero emission. Aquacultural Engineer 29, 165-182.

Tal, Y., Schreier, H., Sowers, K.R., Stubblefield, J., Place, A., Zohar, Y., 2009. Environmentally sustainable land-based marine aquaculture. Aquaculture 286, 28-35.

Timmons, M., Ebeling, J., Piedrahita, R., 2009. Acuicultura en Sistemas de Recirculación. First Edition in Spanish. Editorial CayugaAquaVentures, LLC. Ithaca, NY. 959 pp.

van Rijn, J., 2013. Waste treatment in recirculating aquaculture systems. Aquacultural Engineering 53, 49-56.

Tsukuda, S., Christianson, L., Kolb, A., Saito, K., Summerfelt, S., 2014. Heterotrophic denitrification of aquaculture effluent using fluidized sand biofilters. Aquacultural Engineering 64, 49-59.

Ucisik, A., Henze, M., 2008. Biological hydrolysis and acidification of sludge under anaerobic conditions: The effect of sludge type and origin on the production and composition of volatile fatty acids. Water Research 42, 3729-3738.

van Rijn, J., Tal, Y., Schreier, H.J., 2006. Denitrification in recirculating systems: theory and application. Aquacultural Engineering 34 (3), 364-376.

DTU Aqua – National Institute of Aquatic Resources – is an institute at the Technical University of Denmark. DTU Aqua's mission is to conduct research, provide advice, educate at university level and contribute to innovation in sustainable exploitation and management of aquatic resources. We investigate the biology and population ecology of aquatic organism, aquatic physics and chemical pro-cesses, ecosystem structure and dynamics, taking account of all relevant natural and anthropogenic drivers.

DTU Aqua
National Institute of Aquatic Resources
Technical University of Denmark

Jægersborg Allé 1
2920 Charlottenlund
Denmark
Tlf. +45 35 88 33 00
www.aqua.dtu.dk