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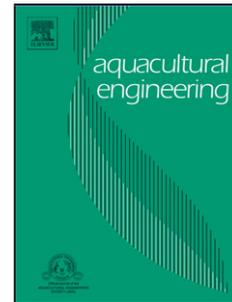
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End-of-pipe denitrification using RAS effluent waste streams: Effect of C/N-ratio and hydraulic retention time

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Highlights

End-of-pipe single-sludge denitrification rate: $125 \pm 15.7 \text{ g NO}_3\text{-N m}^{-3}_{\text{reactor}} \text{ d}^{-1}$

Laboratory sludge hydrolysis: 17-24 % higher VFA compared to sludge basin yield

Effect of C/N-ratio on N-removal rate depended on the hydraulic retention time

A model developed in AQUASIM simulated the on-farm denitrification reactors well

Besides denitrification; indications of anammox and DNRA was observed.

Abstract

Environmentally sustainable aquaculture development requires increased nitrogen removal from recirculating aquaculture systems (RAS). In this study, removed solids from a large commercial outdoor recirculated trout farm ($1000 \text{ MT year}^{-1}$) were explored as an endogenous carbon source for denitrification. This was done by; (1) a controlled laboratory experiment on anaerobic hydrolysis of the organic matter (from sludge cones, drumfilter, and biofilter back-wash), and (2) an on-site denitrification factorial experiment varying the soluble COD (COD_s) / $\text{NO}_3\text{-N}$ ratio from 4 – 12 at hydraulic retention times (HRT) from 50 – 170 min in simple 5.5 m^3 denitrification reactors installed at the trout farm.

The lab-experiments showed that the major part of the readily biodegradable organic matter was hydrolyzed within 14 days, and the hydrolysis rate was fastest the first 24 hours. Organic matter from the sludge cones generated $0.21 \pm 0.01 \text{ g volatile fatty acids (VFA) g}^{-1}$ total volatile solids (TVS), and the VFAs constituted 75% of COD_s . Analogously, 1 g TVS from the drum filter generated $0.15 \pm 0.01 \text{ g VFA}$, constituting 68% of the COD_s . Comparison of the laboratory hydrolysis experiments and results from the on-farm study revealed as a rough

estimate that potentially 17 - 24% of the generated VFA was lost due to the current sludge management.

Inlet water to the denitrification reactors ranged in $\text{NO}_3\text{-N}$ concentration from 8.3 to 11.7 g m^{-3} and COD_5 from 52.9 to 113.4 g m^{-3} ($10.0 \pm 1.2 \square \text{C}$). The highest $\text{NO}_3\text{-N}$ removal rate obtained was at the intermediate treatments; $91.5 - 124.8 \text{ g N m}^{-3}_{\text{reactor}} \text{ d}^{-1}$. The effect of the C/N ratio depended on the HRT. At low HRT, the variation in C/N ratio had no significant effect on $\text{NO}_3\text{-N}$ removal rate, contrary to the effect at the high HRT. The stoichiometric ratio of $\text{COD}_5/\text{NO}_3\text{-N}$ was 6.0 ± 2.4 , ranging from 4.4 (at the high HRT) to 9.3 (at the low HRT). A simple model of the denitrification reactor developed in AQUASIM showed congruence between modeled and measured data with minor exceptions. Furthermore, this study pointed to the versatility of the $\text{NO}_3\text{-N}$ removal pathways expressed by the bacterial population in response to changes in the environmental conditions; from autotrophic anammox activity presumably present at low C/N to dissimilatory nitrate reduction to ammonia (DNRA) at high C/N, besides the predominate “normal” heterotrophic dissimilatory nitrate reduction (denitrification).

Keywords: RAS, denitrification, sludge hydrolysis, volatile fatty acids

1. Introduction

The future increase in aquaculture production in Europe relies on the ability of making it environmentally sustainable in a cost-effective way, a development endorsed by the implementation of the EU Water Framework Directive (Directive 2000/60/EC). In Denmark, this has led to an increasing number of traditional freshwater flow-through trout farms being retrofitted to semi-closed outdoor recirculating aquaculture systems (RAS) called “ModelTroutFarms” (MTF) (Pedersen et al., 2008). The application of RAS in such large commercial trout production systems has demonstrated the potential for combining economically viable production and reduced environmental impact. In the future, aquaculture production in Denmark will be regulated by a licensed maximum allowed nutrient (nitrogen (N), phosphorous (P), organic matter (O)) waste output to the recipient waters. This will give the fish farmer increased incentives to improve farm operation and waste management, enabling increased production without increased environmental impact.

The MTF concept is based on water intake from bore holes and is operated at app. 96% water recirculation. Water treatment consists of particulate matter removal in sludge cones and

drum filters, organic matter removal and nitrification in biofilters, and effluent polishing in constructed wetlands before discharge into the watercourse. Sludge (from sludge cones, drum filter, biofilter back-wash) is stored in sludge basins. From the basins, an overflow of settled water is led into the constructed wetlands. Settled sludge is exported from the MTF for agricultural fertilizing purposes or costly disposal. A two-year measuring campaign on eight MTFs identified N as the nutrient being least retained within the system; 50% of N was removed; 76% of P; 93 % of biological oxygen demand (BOD₅) and 87% of chemical oxygen demand (COD) (Svendsen et al., 2008). Thus, an urge to improve N removal in MTF exists, and consequently formed the main objective of this study.

Dissolved nitrogen is loaded to the water phase in the RAS in the form of total ammonia nitrogen (TAN), representing some 70% of the total dissolved and suspended N-compounds excreted from the protein catabolism in the fish (Dalsgaard and Pedersen, 2011; Kajimura et al., 2004). The mechanisms removing dissolved N from the aqueous environment are either assimilation in bacterial or plant biomass or denitrification. Quantitatively, denitrification is by far the most important removal process in MTF systems. Denitrification is an anoxic microbial process where nitrate in the absence of oxygen is used as electron acceptor for respiration of organic matter (Brock and Madigan, 1991). In the MTF system, the denitrification process is mainly taking place in the constructed wetlands (Svendsen et al., 2008). An overview of the microbial nitrogen cycling processes is schematically represented in Fig. 1. NO₃-N is formed by the aerobic nitrification process (TAN oxidation + nitrite oxidation). The reduction of NO₃-N to gaseous N₂ (denitrification) is a sequence of intermediate steps with NO₂-N being the first intermediate compound in the sequence (Fig. 1). Additionally, dependent on the environmental conditions, two other ways of NO₃-N/ NO₂-N reduction exists: (1) Dissimilatory nitrate reduction to ammonium (DNRA) in excessive reductive environments (high C/N-ratio) (Washbourne et al., 2011), and (2) Anaerobic ammonium oxidation (anammox), an autotrophic process (low C/N-ratio) where ammonium serves as the electron donor for reduction of NO₂-N to N₂ (Jetten et al., 1998).

Bacteria gain less energy by denitrification than by aerobic respiration (van de Leemput et al., 2011), which makes oxygen an important inhibitory regulator of the denitrification process. The relative enzyme activity for the different steps in the denitrification sequence can differ among different bacteria (Tiedje, 1988) and environmental factors (e.g. oxygen level, pH, temperature, energy source/ organic compound availability and nature) will affect denitrification rates (Henze et al., 2002) as well as the regulation of the different enzymatic

steps (Bauman et al., 1996). Complete denitrification without accumulation of toxic intermediates can thus be a challenging operation, if conditions are not properly controlled (van Rijn and Rivera 1990, Lee et al., 2000). The composition of the organic matter affects the denitrification rate as a result of how easily it is metabolized. Small C-compounds (<1000 Da) such as volatile fatty acids (VFA) are readily transportable across the bacterial membrane, whereas larger molecules need to be hydrolyzed by exogenous enzymes before subsequent membrane transport (Ahring, 2003).

Nitrogen removal in specific denitrification reactors has not been extensively used in commercial cold water RAS due to the relatively harmless effect of $\text{NO}_3\text{-N}$ towards fish and a lack of economic incentive to reduce discharge values. In warm water RAS, nevertheless, the advantages of including a denitrification unit, in terms of reduced fresh water intake (/ reduced heating costs) and gain of alkalinity (Otte and Rosenthal, 1979) has been commercialized. Such systems typically rely on an exogenous small molecular weight C-source (e.g. methanol) for the denitrification process, making the process fast and relatively easy to control, because hydrolysis time for transforming a more complex C-source into available small C-compounds can be disregarded. However, a potential cost-efficient and sustainable approach would be to use the endogenous organic matter produced at the RAS as C-source for denitrification (single-sludge denitrification), taking advantage of the concomitant COD reduction (Aboutboul et al., 1995, van Rijn, 1996, Martins et al., 2010). The ratio between available organic matter and nitrate-N (COD/ $\text{NO}_3\text{-N}$ ratio) for denitrification is generally 3 – 6 (van Rijn et al., 2006).

Most of the MTF/ RAS facilities should be able to produce this C/N ratio given appropriate solid organic matter removal technologies. The measured waste output from MTFs demonstrated $\text{COD}/\text{NO}_{2+3}\text{-N} = 6.7\text{-}5.2$ during a two year monitoring project (Svendsen et al., 2008). Klas et al. (2006a) developed a model for single-sludge denitrification in RAS, and demonstrated the feasibility of the model in a lab-scale activated sludge experiment (Klas et al., 2006b).

In this first study reporting on pilot scale single-sludge denitrification applied in commercial MTF systems, we focused on exploiting the existing effluent streams for a controlled and enhanced end-of-pipe denitrification process unit before the constructed wetlands. The easily degradable organic compounds generated by hydrolysis of the organic matter in the sludge thickening basins were used to fuel the denitrification of water discharged from the fish rearing units. The experiment was conducted in 5.5 m^3 tanks. We investigated the effects of

varying the COD₅/NO₃-N ratio from 4 to 12 at hydraulic retention times (HRT) ranging from 50 to 170 min in a full factorial design in order to find optimal combinations for process operation. We consider the two factors are important parameters for future dimensioning and operation of specific single-sludge denitrification reactors. Degradation properties of the organic matter were characterized in a separate laboratory hydrolysis experiment.

2. Materials and Methods

2.1. Experimental set-up at the fish farm

Two identical 5.5 m³ cylindrical PE-tanks (height 3 m, diameter 1.7 m) with conical (60° slope) bottom were operated as up-flow single-sludge denitrification reactors in a duplicate set-up. Nitrate-rich outlet water (12.7-17.6 g NO₃⁻-N m⁻³) from a commercial (1,000 MT/y), outdoor trout RAS (described in Suhr and Pedersen (2010)) was supplied to the experimental reactors by way of a side-stream passage. An overflow stream from the sludge basin at the RAS was used as organic carbon source for the denitrification process. The two inlet streams were mixed just prior to the inlet at the bottom of the up-flow reactors.

Flows were continuously monitored by electronic flowmeters (IFC010, KROHNE, Duisburg, Germany) to support calculation of the ratio of NO₃⁻-N-containing RAS effluent and C-containing water from the sludge basin.

The denitrification reactors had been operating for more than three months prior to the experiment.

2.2. Sludge hydrolysis

The sludge basin was fed by three organic matter waste streams from the RAS facility; (1) flush water containing settled faecal matter and uneaten feed pellets from sedimentation traps/sludge cones, (2) flush water with drum filter retained particulate matter, and (3) biofilter back-wash water (Table 1). The sludge cones were emptied every 48 hrs. Two of the 12 biofilter sections were backwashed daily, which in effect gave a weekly backwash frequency and a daily backwash discharge to the sludge bed. The drum filter was in continuous operation. Degradation properties of the organic matter from these different streams were characterized by a separate 16 day anaerobic hydrolysis experiment.

Samples were collected at the RAS as a single sampling event, extracted approximately halfway through the flushing event, e.g. if the flushing took approximately 12 min, the sampling began after 5 min. The drum filter waste sample, however, was taken from a temporary sludge-thickening device receiving the effluent waste stream. From each discharge stream a 20 l sample was collected, cooled on ice, and transported directly to the laboratory. Each sample was distributed in triplicate 2 l glass bottles sealed with rubber stoppers, and incubated at room temperature ($20 \pm 2^\circ\text{C}$) with equal, continuous complete stirring by use of magnetic stirrers. Supernatant water was discharged prior to incubation if a well-defined phase- separation had occurred in the raw samples, and the up-concentration factor measured as; volatile suspended solids (VSS) content in sample/VSS content in raw sample, was noted. VSS was measured according to Danish Standard (DS 207). Produced gas was vented out through a one-way valve inserted in a tube through the rubber stopper. A second tube in the stopper was extended into the water phase, from which samples of 50 ml were withdrawn. Inert nitrogen gas was used to purge the sludge solution backwards in the sampling tube when sampling sessions were ended.

Samples were taken at day 0, 1, 2, 4, 6, 8, 10, 14, and 16. Analysis of the samples comprised measurement of pH, total chemical oxygen demand (COD_T) (Hach Lange test kit no. LCK 514), soluble COD (COD_S) (Hach Lange, LCK 414), TAN (Hach Lange, LCK 305), and volatile fatty acids (VFA, Hach Lange, LCK 365). Total volatile solids (TVS) were measured on raw samples, and in the hydrolysis experiment on concentrated samples at day 0 and 16, by oven drying according to Danish Standard (DS 204). The samples for chemical analysis were centrifuged ($4500 \times g$, 10 min, 5°C) prior to filtration ($0.45 \mu\text{m}$) with exception of total COD samples. The Hach Lange test kit (LCK 365) method used for measuring the VFA content quantifies all the acids as acetate. A factor of 1.07 was used to convert acetate into acetate-COD units based on the stoichiometry of acetate oxidation ($\text{g O}_2 \text{g}^{-1} \text{acetate}$);

$$\text{CH}_3\text{COOH} + 2 \text{O}_2 \rightarrow 2 \text{CO}_2 + 2 \text{H}_2\text{O}$$

2.3. On-farm denitrification experiments: Design, sampling, and chemical analysis

The experimental design and subsequent response surface analysis was made with Unscrambler[®] 9.1 (Camo Software, Norway).

The C/N ratio ($\text{COD}_S / \text{NO}_3^- \text{-N}$) of the inlet water to the experimental denitrification reactors was varied from 4 to 12 and the hydraulic retention time (HRT) from 50 to 150 min

according to a full factorial design with two replicate runs of the intermediate treatment (Table 2). The experimental order was randomised and the intermediate runs were conducted with a 7 day interval. For data processing, the independent variables (C/N-ratio and HRT) were the measured data (and not the designed values) as these were not identical in all settings due to practical restrictions (Table 2). Furthermore, data from 5 additional runs in the same setup (C/N 5.6 – HRT 96; C/N 7.5 – HRT 159; C/N 11 – HRT 197; C/N 15.8 – HRT 109, C/N 16 – HRT 188), representing a 14 week period in all, were included in the dataset for response surface modelling to strengthen the model.

Student's t-tests (2-tailed, unpaired, equal variances) were performed in Excel 2010 (Microsoft Office).

Water temperature was 10.0 ± 1.2 °C (n=14) during the experiments.

Acclimatization time before sampling was minimum 3 times the HRT of the treatment. Replicate samples from reactor inlet and outlet were taken at 10 min interval from both reactors. The samples were immediately filtered (0.45 µm) and analyzed for TAN by the indophenolblue method with salicylate (Danish Standard DS 224), NO₂-N by the diazotization with sulfanilamide and coupling to N-(1-naphyl)-ethylendiamine method (DS 223), and NO₃-N by reaction with 2, 6-dimethylphenol (ISO 7890/1). Chemical oxygen demand (COD_s) was determined by Merck test kit (Merck KGaA, Darmstadt, Germany), which was modified from ISO 15705. Alkalinity was measured by titration to pH 4.5 by 0.1 M hydrochloric acid. Temperature, oxygen content and pH were monitored with Hach HQ40d instruments (Hach Lange GmbH, Düsseldorf, Germany).

Content of VFA (Hach Lange test kit LCK 365) in the overflow stream from the sludge basin was also monitored at each experimental run.

2.4. Modeling of the denitrification reactors

The computer program AQUASIM 2.1 (Reichert, 1994) for identification and simulation of aquatic systems was used for modeling the single-sludge denitrification reactor. The expression used for describing the denitrification rate was according to Henze et al. (2002):

$$r = \mu_m * ((C_{NO_3} / (C_{NO_3} + K_{SNO_3})) * (COD_{bio} / (COD_{bio} + K_{SCOD}))) * X$$

where; r: Denitrification rate (g N m⁻³ d⁻¹); μ_m : Maximum specific growth rate of heterotrophs (d⁻¹); C_{NO₃}: Nitrate-N concentration (g m⁻³); K_{SNO₃}: Half-saturation constant for NO₃-N (g N m⁻³); COD_{bio}: Biodegradable COD (g m⁻³); K_{SCOD}: Half-saturation constant for COD_{bio} (g COD m⁻³); X: Biomass concentration (g COD m⁻³).

The stoichiometric coefficients of the variables were:

$$C_{\text{NO}_3}: - (1-Y)/(2.86 * Y)$$

$$\text{COD}_{\text{bio}}: - 1/Y$$

X: 1 (biomass concentration in the tank equals to the biomass concentration in the inlet, because the HRTs employed were relatively short (0.8 - 3.0 h) compared to microbial doubling times (min. 3.7 h)).

The yield constant, Y, was set to 0.5 g COD g⁻¹COD, K_{SCOD} to 10 g COD m⁻³, K_{SNO₃} to 0.35 g NO₃-N m⁻³, and μ_m to 4.5 d⁻¹. All values derived from Henze et al. (2002).

The biodegradable COD (COD_{bio}) was approximated by the soluble COD, COD_S.

The biomass (X) was approximated by 0.5 * the particulate COD fraction, and total COD (COD_T) was set to 3 * soluble COD (COD_S), thus; X = (3 * COD_S - COD_S) * 0.5.

The 5.5 m³ tank was modeled as a completely mixed flow reactor (CMFR) because data for hydraulic flow characterization were not available. The inlet flows and inlet substrate concentrations were inserted in the model according to Table 2.

3. Results

3.1. Sludge hydrolysis experiment

Anaerobic hydrolysis of two different organic waste streams - sludge cones and drum filter - from the RAS farm showed similar degradation patterns independent of origin (Fig. 2 and 3). The quantity of organic matter sampled from the biofilter back-wash water was too low to generate a reproducible result of the hydrolysis from this source (Table 1).

Organic matter from the sludge cones generated 0.21 ± 0.01 g VFA g⁻¹ TVS, and the VFAs constituted 75% of COD_S. Drum filter retained organic matter generated 0.15 ± 0.01 g VFA g⁻¹ TVS and VFA constituted 68% of the COD_S (Table 1).

The highest VFA generation rate was recorded at the start of the experiment (Fig. 3). This was correlated to the pH readings that showed the sharpest drop during the first 24 h (Fig. 2). No increase in VFA was recorded from day 14 to 16 (Fig. 3).

An increase in TAN during hydrolysis was measured, and the production was closely correlated to VFA generation (Fig. 4). The data showed a production of 0.08 and 0.11 g TAN g⁻¹ VFA-COD from the sludge cone (R²=0.99) and the drum filter (R²=0.96) sludge, respectively.

During the hydrolysis period (day 0-16), the proportion of COD_S to COD_T increased from $1.5\% \pm 0.1$ to $15.1\% \pm 0.4$ and from $3.7\% \pm 0.1$ to $15.1\% \pm 1.1$ for sludge cones and drum filter sludge, respectively.

3.2.1. Performance of the single-sludge denitrification reactors: NO_3-N removal

The highest N removal rate was obtained at the intermediate treatments and reached $91.5 - 124.8 \text{ g } NO_3-N \text{ m}^{-3}_{\text{reactor}} \text{ d}^{-1}$ (Table 3). High nitrate removal ($95.4 \text{ g } NO_3-N \text{ m}^{-3}_{\text{reactor}} \text{ d}^{-1}$) was also monitored at the high C/N - high HRT treatment, comparable to the removal range at the intermediate runs. However, the high C/N - high HRT treatment generated a significant amount of TAN (Table 3), a unique feature of this treatment. The experiment showed that the effect of the C/N ratio depended on the HRT. At low HRT, the variation in C/N ratio had no significant effect on NO_3-N removal rate (Table 3), contrary to the effect at the high HRT (by t-tests).

The average NO_3-N concentration varied from 8.3 to 11.7 g m^{-3} in the inlet water and the average COD_S varied from 52.9 to 113.4 g m^{-3} in the experimental runs (Table 2). Predicted outlet values from the model simulations of the single-sludge denitrification were in the same order as the experimental data, despite of the very simple model and rough assumption of flow characteristics (completely mixed) and of the values applied for the constants. Only few treatments were poorly simulated ($6.04 \text{ g } NO_3-N \text{ m}^{-3}$ measured in the outlet versus 3.3 g m^{-3} modeled in the High C/N - Low HRT treatment, and $31.2 \text{ g } COD_S \text{ m}^{-3}$ measured in the outlet versus 12.5 g m^{-3} modeled in the Low C/N - High HRT treatment (Table 2)). The cause of these major deviations will be further treated below (3.2.2. *TAN, NO_2-N , and alkalinity changes* and in 4. Discussion).

Performing a multivariate response surface analysis of the data rendered a maximal nitrate removal of $98.6 \text{ g } NO_3-N \text{ m}^{-3}_{\text{reactor}} \text{ d}^{-1}$ at $C/N=9.4$ and $HRT=111 \text{ min}$ ($R^2=0.84$, and a multiple correlation coefficient 0.92) (Fig. 5). Thus, the multivariate data analysis indicated that the optimal setting at the running conditions was close to the intermediate levels. Besides the main effects (C/N and HRT) the interaction and squared terms were included in the model. Only the squared terms were significant, indicating a non-linear effect of C/N and HRT, in accordance with figure 5.

Overall, the stoichiometric ratio of $\text{COD}_S/\text{NO}_3\text{-N}$ measured experimentally was 6.0 ± 2.4 (average \pm s.d., $n=12$), ranging from 4.4 ± 0.8 to 9.3 ± 2.0 at the high and low HRT, respectively. Inlet pH during the experiment was 7.00 ± 0.08 (average \pm s.d., $n=12$), ranging from 6.92 ± 0.01 to 7.07 ± 0.03 at high C/N and low C/N, respectively.

The VFA-COD concentration in the overflow stream of the sludge basin feeding the denitrification reactors during the experiment was on average $94 \pm 18 \text{ mg l}^{-1}$ ($n=6$) making up $51 \pm 6\%$ of the sCOD content.

3.2.2. Performance of the single-sludge denitrification reactors: TAN, $\text{NO}_2\text{-N}$, and alkalinity changes

The reactors removed TAN as expected with (low) O_2 content in the inlet waters at all settings (Table 3), except at high C/N – high HRT where TAN was produced. This unique net production of TAN was correlated with a considerable higher alkalinity production than expected if only dissimilatory nitrate reduction to N_2 was considered (Table 3). At low C/N – high HRT conditions, on the other hand, increased TAN removal was observed, and an alkalinity consumption was measured, which was contrary to the theoretically calculated alkalinity production based upon mere nitrate reduction to N_2 (Table 3).

$\text{NO}_2\text{-N}$ values showed the relatively greatest variability between the two replicate reactors (Table 3) in accordance with the intermediate nature of this N-compound. The highest $\text{NO}_2\text{-N}$ production levels (Table 3) coincided with the two treatments with poor concordance between modeled and measured output values (Table 2).

4. Discussion

4.1. Sludge hydrolysis experiment

When comparing the two sludge types, the drum filter waste had a lower pH-value (Fig. 2) and a higher VFA content (Fig. 3) at start (day 0), than the sludge cone waste. This could be due to either (1) generally older or readily degradable material with hydrolysis and fermentation processes already in progress, or (2) smaller particles rendering higher hydrolysis rate affecting the material even before start up in the laboratory (despite the cooled state during transport), or a combination of both. From hydrolysis day 8 and onwards, a

tendency to relative stagnation in VFA and COD_S generation from drum filter waste compared to sludge cone waste was observed (Fig. 3). The marginal difference observed in TAN generating rate between the two waste sources (Fig. 4) might indicate that drum filter particles contained more protein than particles from sludge cones. This effect, however, might also relate to relatively older material, since protein degradation has been found to be more dependent on sludge retention time than carbohydrate and lipid degradation (Elefsiniotis and Oldham, 1994). The sludge cone waste exhibited faster settling properties than the drum filter waste, probably due to more dense and larger sized particles compared to the drum filter waste. Anyway, the stirring during the experiment probably disintegrated the particles into more homogenous sizes, and the initial physical difference between the samples did not seem to affect the measured sample characteristics (pH, VFA, COD_S, and TAN) to a greater extent than the points mentioned above. The pH dropped below 6 on the first day of hydrolysis (Fig. 2). At this pH, loss of VFA to methane production is most likely negligible since methanogenic bacteria are considered to be inhibited at pH below 6-5.5 (Henze et al., 2002).

The organic matter was sampled from the RAS farm according to the routines for waste discharge and as a single sampling event (section 2.2. *Sludge hydrolysis*). More extensive experiments will have to be conducted to fully elucidate differences in sludge types and time effects on degradation.

On-farm analysis of the overflow stream from the sludge basin to the denitrification reactors during the experiment showed that VFAs constituted 51 ± 6 % of the COD_S. In the laboratory, the VFAs made up 68 and 75 % of the COD_S from drum filter and sludge cone waste, respectively (Table 1). This indicates that a considerable amount (about 17 - 24 %) of potential VFAs were unexploited or simply lost by aerobic heterotrophic growth, dissimilatory nitrate reduction to ammonia (or N₂), and/or methanogenic activity, because the current sludge storage basin management was not optimized for generating VFAs for denitrification.

The greatest VSS reduction measured after 16 days of hydrolysis was 32% (in sludge cones), and consequently more than 2/3 of the organic matter was very slowly biodegradable. Klas et al. (2006a) reported a biodegradability in all likelihood above 80% for marine aquaculture sludge (in a 15 days period), and up to 99.6% COD removal has been found in RAS sludge

digestion (HRT 6-8 days at 30⁰ C) for methane production (Mirzoyan et al., 2010). This

suggests that optimization of biomass and hydrolysis conditions could further improve the biodegradability of the organic matter collected at the farm.

4.2.1. Single-sludge denitrification experiment – NO₃-N removal rate

This study was conducted on a commercial RAS farm, imposing a substantial source of variation on the data from farm-management and natural dynamics, which is partly reflected in the observed variability from the duplicate denitrification reactors. Removal rates were calculated as concentration differences (between denitrification reactor inlet and outlet) times the flow rate. This makes the impact from analytical measurement error relatively more susceptible to low HRT treatments (as the lower difference in absolute concentrations yields higher coefficients of variation). These technicalities should be kept in mind when evaluating the results (Table 2 and 3).

The maximum denitrification rate in the reactor was $125 \pm 15.7 \text{ g NO}_3\text{-N m}^{-3}_{\text{reactor}} \text{ d}^{-1}$ (or $60 \pm 6.0 \text{ g NO}_3\text{-N kg}^{-1}\text{TVS d}^{-1}$) when utilizing the organic matter from the overflow of the sludge storage basin as carbon source for the process. In a comprehensive review, van Rijn et al. (2006) reported denitrification rates in aquaculture systems ranging from 24-3984 g NO₃-N m⁻³ d⁻¹. This wide range reflected differences in system configurations, operational parameters, management, etc.. The maximum rate measured in the present study was more than doubled when polyethylene netshaped (Bioblok[®], 200 m² m⁻³) fixed-bed media were inserted in the reactors (Suhr et al., unpublished results), indicating that retention of suspended solids and heterotrophic biomass in the reactors and altered flow characteristics could considerably increase N-removal efficiency. In accordance with this, experimental scale fluidized bed and polymer media filters have shown high NO₃-N removal rates (1039-1848 g NO₃-N m⁻³ d⁻¹) in aquaculture systems (Boley et al., 2000, Shnel et al., 2002, Gelfand et al., 2003). Increased surveillance and diligent sludge removal management, however, are required in such filter types.

The NO₂₊₃-N removal rate measured in the constructed wetlands of the MTFs was on average 1.5 – 1.9 g N m⁻² d⁻¹ (Svendsen et al., 2008). At an average depth of 0.9 m in the wetlands this

corresponds to a removal of $1.7 - 2.1 \text{ g N m}^{-3} \text{ d}^{-1}$. The maximum volumetric removal rate of $125 \pm 15.7 \text{ g NO}_3\text{-N m}^{-3}_{\text{reactor}} \text{ d}^{-1}$ measured in this study makes the N-removal process 60 - 75 times more efficient in the reactors than in the constructed wetlands. However, the C/N-ratio was somewhat higher in the maximum N-removal reactor treatment ($\text{COD}_s/\text{NO}_3\text{-N} = 6.9$) compared to the average ratios in the inlet to the constructed wetlands ($\text{COD}/\text{NO}_{2+3}\text{-N} = 5.5$). Nevertheless, the results of this study suggest strongly that a more dedicated sludge treatment would be profitable if a MTF was to implement a controlled single-sludge denitrification unit prior to the constructed wetlands. In an optimized sludge digestion unit fueling the denitrification reactor, proper hydraulic mixing should be sustained. Incoming “pure” flush water (containing high oxygen but no nitrate nor considerable organic matter) should be bypassed straight into the constructed wetlands or as a minimum up-concentrated before entering the sludge digestion unit. A drawback of using single-sludge denitrification technology is the more soluble state of the wasted sludge and release of nutrients (TAN, PO_4^{2-}) from it (Klas et al., 2006a, Conroy and Couturier, 2010). A clarifier unit and further removal of excess nutrients could potentially remedy this and lead to successful operation of the whole RAS system.

4.2.2. *Single-sludge denitrification experiment – Environmental effects on microbial N-cycle processes*

In all the denitrification experimental runs, a little nitrifier activity was assumed, as oxygen was present in the inlet (Table 3) and some TAN removal was measured. However, the amount of oxygen consumed by nitrifiers compared to heterotrophs was not possible to quantify from the data obtained. Similarly, it was not possible to distinguish whether the measured $\text{NO}_2\text{-N}$ removed or formed originated from incomplete/partial nitrification or incomplete denitrification, as such quantification would require tracer marked N-compounds. Consequently, the intermediate $\text{NO}_2\text{-N}$ compound pool was disregarded when calculating the theoretical alkalinity removal by denitrification in Table 3, as well as in the reflections below.

At high HRT, the response of the microbial N-transformation pathways to the differentiated environmental conditions imposed by the varied C/N-ratio was distinct.

The TAN production observed at the high C/N – high HRT treatment was most likely due to dissimilatory nitrate reduction to ammonia (DNRA). The alkalinity production, being higher than expected when only dissimilatory nitrate reduction to N_2 was considered (Table 3),

confirmed that additional alkalinity was produced probably via DNRA, as DNRA produces about twice the alkalinity as denitrification (van de Leemput et al., 2011).

Conversely, at the low C/N – high HRT treatment, an increased TAN removal was observed, along with a net alkalinity consumption (Table 3). If all TAN was oxidized by nitrifiers alkalinity consumption would have been $2.8 \text{ eqv m}^{-3} \text{ d}^{-1}$, and with all $\text{NO}_3\text{-N}$ removed by heterotrophic denitrification (producing $2.6 \text{ eqv alkalinity m}^{-3} \text{ d}^{-1}$, Table 3), the overall alkalinity consumption would be a total of $0.2 \text{ eqv m}^{-3} \text{ d}^{-1}$. (Even if all $\text{NO}_2\text{-N}$ produced was included in the nitrified N-pool, a maximal net consumption would be $1.6 \text{ eqv alkalinity m}^{-3} \text{ d}^{-1}$.) This leaves a relatively large gap up to the $2.6 \text{ alkalinity measured}$ (Table 3). The anammox process ($\text{TAN} + 1.32 \text{ NO}_2 + 0.066 \text{ HCO}_3^- + 0.13 \text{ H}^+ \rightarrow 1.02 \text{ N}_2 + 2.03 \text{ H}_2\text{O} + 0.26 \text{ NO}_3^- + 0.066 \text{ CH}_2\text{O}_{0.5}\text{N}_{0.15}$) produces $0.064 \text{ eqv alkalinity mol}^{-1} \text{ TAN}$ (Kunenen, 2008). One possible hypothesis, that could make the alkalinity measured match the N-transformations, could be the presence of the anammox process removing some part of the $\text{NO}_3\text{-N}$ (via $\text{NO}_2\text{-N}$) and TAN. This anammox hypothesis is furthermore supported by the AQUASIM model, since the model showed a remarkable large discrepancy for this particular run between the modeled COD_5 output versus the 2.5 times higher measured COD_5 output (Table 2). Thus, in the most reductive environment (high C/N) indications of DNRA activity was detected, whereas in the least reductive environment (low C/N) indications of autotrophic anammox activity was noted (Table 3). This effect of C/N-ratio on activation of alternative $\text{NO}_3\text{-N}$ removal pathways was not detected at the low HRT, probably because less extreme environments were induced at such short retention time (50 min).

The growth rate of anammox bacteria is very low ($0.1\text{-}0.05 \text{ day}^{-1}$, i.e. doubling time 1 - 2 weeks), which makes the occurrence of these bacteria in normal heterotrophic denitrifying wastewater processes unlikely (van Loosdrecht and Jetten, 1998). However, since the MTF sludge storage basin was not operated as a completely mixed system, it had been continuously in use (> 2 years), and it was the source feeding organic matter and biomass into the denitrifying reactors, it seems reasonable to assume that such bacteria existed in the environment. Anammox bacteria have been identified in (marine) seabream RAS environment (solid waste, faeces, and fish intestines) (Lahav et al., 2009), and the contribution of the anammox process in denitrifying filters has previously been quantified to 15% of the total removal (Tal et al., 2009).

4.2.3. Single-sludge denitrification experiment – Modeling of the denitrification reactors

A very simple model was formulated in AQUASIM, only describing the heterotrophic denitrification process and using the yield constant and half-saturation constants normally applied in wastewater treatment systems. The modeled outlet values were within 49 - 119 % of measured $\text{NO}_3\text{-N}$ (average 77 ± 26 %) and within 40 - 98 % of measured COD_s values (average 77 ± 21 %) (Table 2).

At high HRT, the effect of increasing the C/N-ratio from low to high level on $\text{NO}_3\text{-N}$ removal was measured (as well as modeled, (Table 2)), as an approximate doubling of the rate (Table 3). However, at low HRT, a similar C/N-ratio effect on $\text{NO}_3\text{-N}$ removal was modeled but not measured. This was due to the poorly simulated Low HRT - High C/N-ratio treatment (Table 2). Whether the divergence was caused by distortion in effect between the CMFR modeled and the actual unknown flow-type at this treatment, or was caused by an unknown sludge basin management-inflicted effect, cannot be determined from the available data. It is well-known, however, that the type of hydraulic flow regimen in biological filters is an important factor for efficiency and performance (Chen et al., 2006).

Overall, the COD_s model simulations were satisfactory, apart from the High HRT - Low C/N-ratio treatment, presumably having autotrophic anammox activity. The modeled outlet COD_s values were all generally lower than experimentally measured. This could indicate that the approximation of biomass or yield factor was marginally overestimated. The estimation of biomass was based upon the measured COD_s values (section 2.4. *Modeling of the denitrification reactors*) implicating a relatively great uncertainty about the estimate. A more precise assessment could have been done by measurements in separate (lab) experiments on subsamples from the reactors.

Denitrifying pilot-scale upflow sludge blanket reactors in pilot scale tilapia RAS are operated at flow velocities of $0.3 - 0.6 \text{ m h}^{-1}$ (Eding et al., 2009) or $0.32 - 0.33 \text{ m h}^{-1}$ (Martins et al., 2009) to ensure a high sludge retention time (SRT) relatively to a short water HRT, as sludge hydrolysis and VFA generation is an integrated part of these types of reactors. In this study, the up-flow velocity ranged from $0.9 - 3.0 \text{ m h}^{-1}$ (high HRT $0.9\text{-}1.0 \text{ m h}^{-1}$; low HRT $2.9 - 3.0 \text{ m h}^{-1}$; intermediate HRT 1.4 m h^{-1}). According to the guidelines mentioned by Eding et al. (2009) this could lead to a loss of the major part of the particulate matter in the reactors. It seems reasonable to assume that this indeed happened, since the experimental data showed relatively acceptable fit with the CMFR model applied (HRT = SRT in the CMFR model). The response surface plot (Fig.5) illustrates well that the highest removal rate was measured at the intermediate levels.

5. Conclusions

Further reduction of nitrogen discharge from Danish Model Trout Farms (MTF) requires more efficient denitrification than presently obtained in the constructed wetlands. This study showed that controlled single-sludge denitrification is a feasible option and a rational next step of development for Danish MTFs. Hydrolysis experiments with MTF-generated sludge indicated that potentially 17 - 24 % of the volatile fatty acids (VFA) produced in the MTF sludge storage basin was lost due to the current sludge management practice. Optimizing sludge handling in order to fully take advantage of the biodegradable organic matter will be a central parameter for successful single-sludge denitrification.

In the single-sludge denitrification experiment investigating C/N-ratios from 4 – 12 at hydraulic retention times of 50 – 170 min, the maximum denitrification rate was found to be $125 \pm 15.7 \text{ g NO}_3\text{-N m}^{-3}_{\text{reactor}} \text{ d}^{-1}$ (corresponding to $60 \pm 6.0 \text{ g NO}_3\text{-N kg}^{-1} \text{ total volatile solids (TVS) d}^{-1}$) at the intermediate settings C/N = 6.9 and HRT = 98 min.

The experiment showed that the effect of the C/N-ratio was dependent on HRT. Increased C/N-ratio induced a higher NO₃-N removal rate at high HRT. At low HRT, however, this effect was not observed. Furthermore, this study pointed towards versatile bacterial NO₃-N removal pathways in response to changes of environmental conditions; from indications of autotrophic anaerobic ammonia oxidation (anammox) activity at low C/N to dissimilatory nitrate reduction to ammonia (DNRA) at high C/N-ratio.

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Table 1. Characteristics of the three different organic matter waste stream samples taken from the commercial RAS farm and used for a 16 days laboratory hydrolysis experiment (average \pm s.d., n=3).

	Start, day 0		End, day 16		
	Up-conc. ^a	TVS ₀ (g kg ⁻¹)	TVS ₁₆ (g kg ⁻¹)	COD _S ^b /TVS ₀ (g g ⁻¹)	VFA ^b /TVS ₀ (g g ⁻¹)
Sludge cones	4.8	19.74 \pm 0.36	16.84 \pm 0.70	0.28 \pm 0.01	0.21 \pm 0.01
Drum filter	-	33.16 \pm 0.05	30.34 \pm 0.34	0.22 \pm 0.02	0.15 \pm 0.01
Back-wash ^c	4.3	1.04 \pm 0.05	1.00 \pm 0.07		

^aUp-concentration factor: VSS content of sample (when supernatant water had been tapped off)/ VSS content of raw sample

^bProduced during the hydrolysis period (content day 16 – content day 0)

^cThe initial TVS₀ content was too low to generate reproducible results during hydrolysis, and was subsequently withdrawn from further analysis

Table 2. The experimental runs for the experiment investigating C/N ratio (as COD_S / NO₃-N) and hydraulic retention time (HRT) from 4 – 12 and 50 – 170 min, respectively (**H**: High, **L**: Low, **I**: Intermediate) in two 5.5 m³ reactors. Measured (out) values of NO₃-N and COD_S from the reactors (average \pm s.d., n=2) are listed versus modeled (m. out) values.

Experimental runs		Flow Q (m ³ d ⁻¹)	NO ₃ -N (g m ⁻³)					
C/N	HRT (min)		In	out	m. out*	in		
L	4.6 \pm 0.3	L	49 \pm 0.2	160.64 \pm 0.79	11.62 \pm 0.20	9.82 \pm 0.37	9.2	52.9 \pm 2.5
H	12.2 \pm 0.3	L	50 \pm 0.2	159.56 \pm 0.74	8.29 \pm 0.17	6.04 \pm 0.20	3.3	101.4 \pm 0.0
L	4.2 \pm 0.9	H	167 \pm 0.6	47.52 \pm 0.17	11.60 \pm 0.94	6.98 \pm 1.96	5.3	48.8 \pm 6.9

H	10.7 ± 0.1	H	148 ± 1.6	53.44 ± 0.57	10.60 ± 0.10	0.77 ± 1.03	0.4	113.4 ± 0.2
I	8.5 ± 0.4	C	104 ± 1.1	76.40 ± 0.79	9.18 ± 0.31	2.59 ± 0.30	1.8	77.9 ± 0.9
I	6.9 ± 0.0	C	98 ± 0.2	80.76 ± 0.17	11.67 ± 0.08	3.17 ± 1.13	3.8	80.6 ± 0.6

*Value in the outlet according to model simulations.

Table 3. Removal rate of NO₃-N, TAN, and NO₂-N measured at the different experimental runs. The measured removal rate of alkalinity (negative removal= production) is shown versus the calculated alkalinity removal if all nitrate was removed by dissimilatory reduction to N₂. (Average ± s.d., n=2)

Experimental runs		Inlet O ₂ (g m ⁻³)	Removal rate (g m ⁻³ reactor d ⁻¹)					
C/N	HRT (min)		NO ₃ -N	TAN	NO ₂ -N	All		
L	4.6 ± 0.3	L	49 ± 0.2	3.55 ± 0.29	52.6 ± 16.3	2.1 ± 1.4	-5.5 ± 6.2	-3
H	12.2 ± 0.3	L	50 ± 0.2	2.16 ± 0.13	65.3 ± 10.6	4.5 ± 8.7	-8.9 ± 7.6	-3
L	4.2 ± 0.9	H	167 ± 0.6	2.80 ± 0.28	39.9 ± 8.7	19.7 ± 2.8	-9.6 ± 7.7	2
H	10.7 ± 0.1	H	148 ± 1.6	3.43 ± 0.03	95.4 ± 8.1	-5.4 ± 4.3	3.8 ± 5.3	-9
I	8.5 ± 0.4	I	104 ± 1.1	2.62 ± 0.13	91.5 ± 7.5	4.4 ± 5.3	-1.9 ± 2.9	-6
I	6.9 ± 0.0	I	98 ± 0.2	4.02 ± 0.04	124.8 ± 15.7	6.4 ± 3.1	2.1 ± 6.1	-10

*DN: Denitrification. Alkalinity removal calculated as - 0.91 eqv. mol⁻¹ N (Henze et al., 2002).

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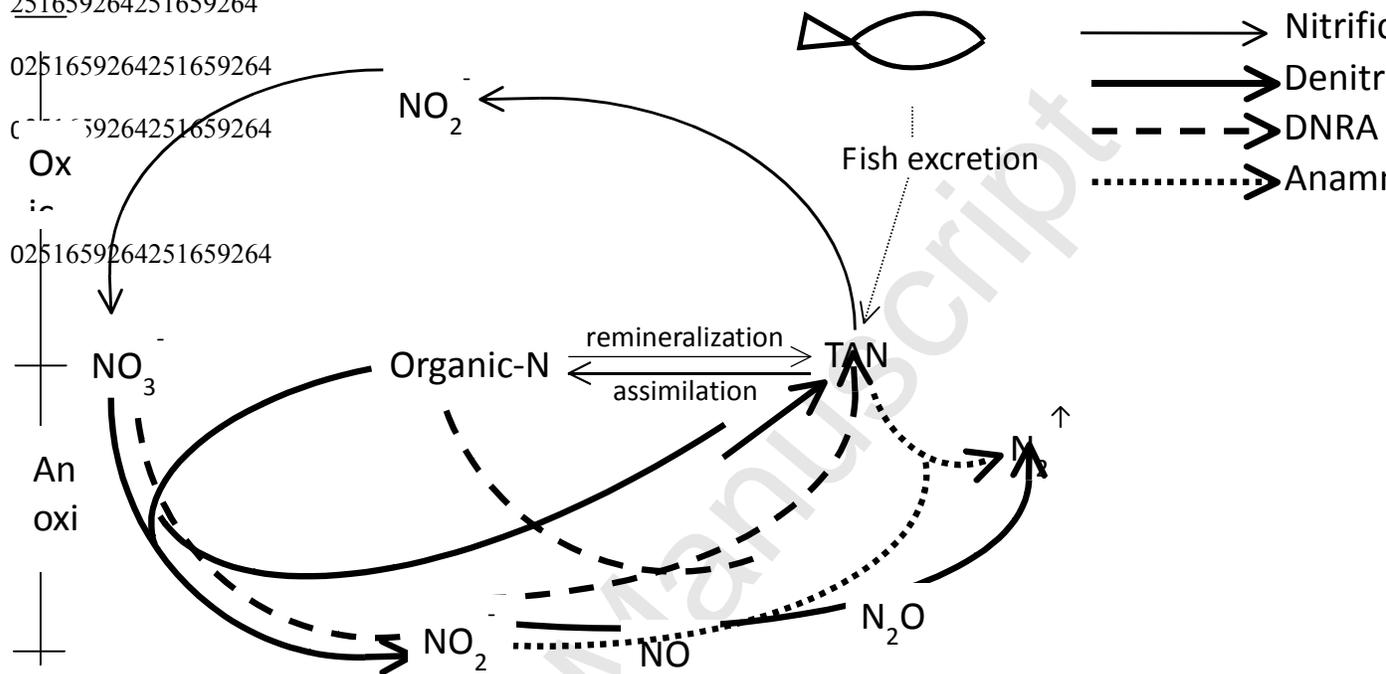
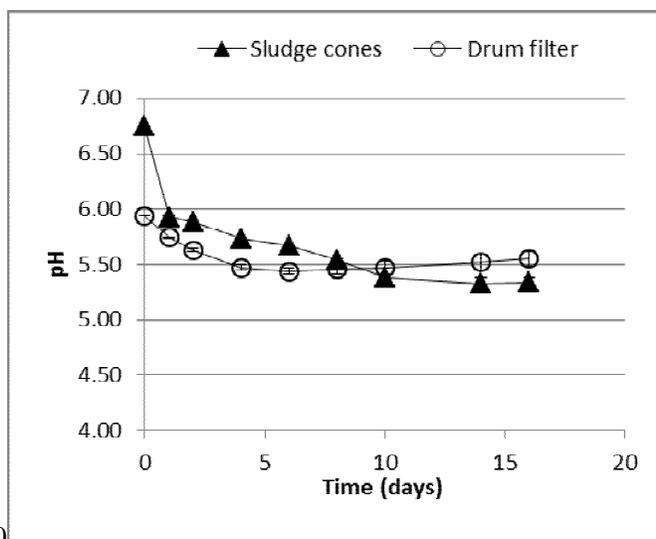
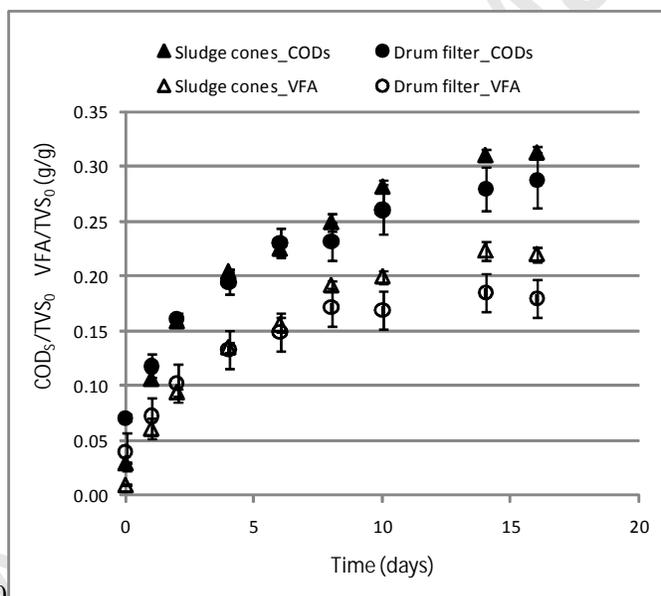
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Figure 1. Microbial Nitrogen cycling processes. Modified from Lam et al., 2009.



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Figure 2. Measured pH values during hydrolysis. The initial drop reflects the high initial generation rate of high volatile fatty acids. (Average \pm s.d., n=3).



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Figure 3. Production of soluble COD (COD_s) and volatile fatty acids (VFA) during hydrolysis. Data are normalized by division by organic matter content on day 0 (TVS_0). (Average \pm s.d., n=3).

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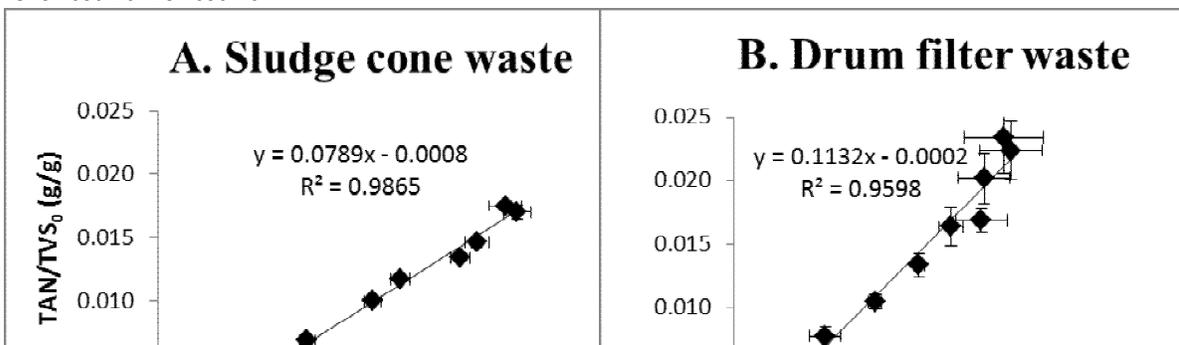


Figure 4. Correlation of TAN and VFA production during anaerobic hydrolysis of waste from sludge cone (A) and drum filter (B). (Average \pm s.d., n=3).

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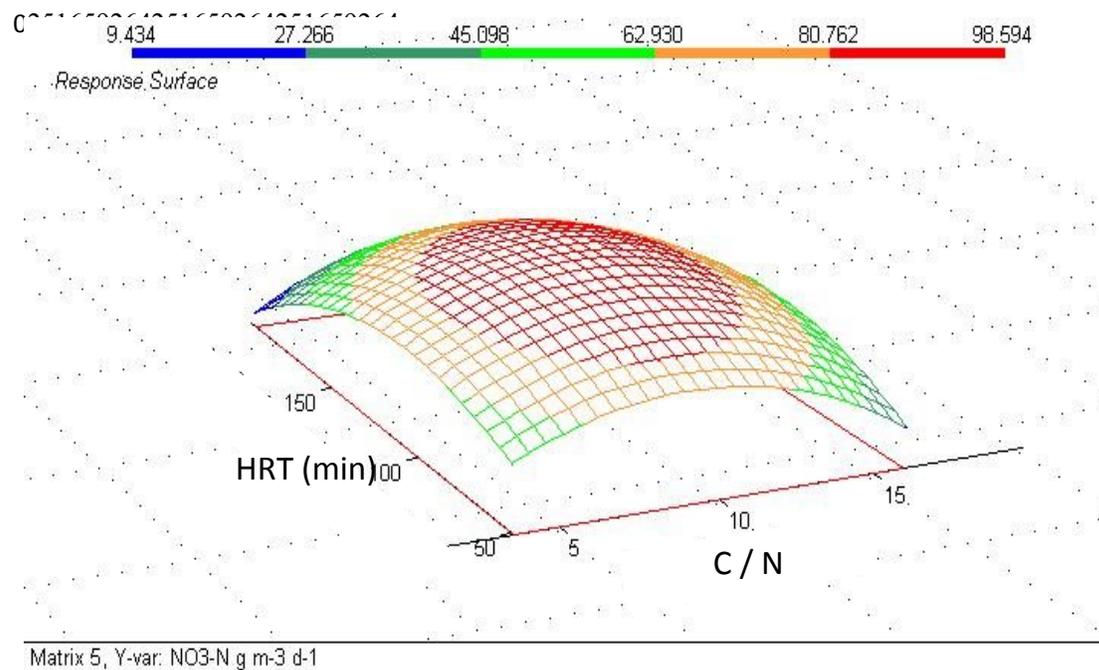


Fig. 5. Response Surface analysis of the effect of hydraulic retention time (HRT) and C/N-ratio on $\text{NO}_3\text{-N}$ removal ($\text{g m}^{-3} \text{d}^{-1}$).