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Changes in microbial water quality in RAS following altered feed loading

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ABSTRACT

Intensive recirculating aquaculture systems (RAS) with its hyper-eutrophic water offer ideal conditions for bacterial growth, abundance and activity, potentially affecting fish and system performance. Feed composition and feed loading in particular will have significant impact on organic and inorganic nutrients available for microbial growth in RAS. How these nutrient inputs affect and regulate bacteria in RAS water is, however, unclear. To investigate this relationship and the associated water quality dynamics, the effects of altered feed loading on microbial water quality in RAS was studied.

The study included six independent, identical pilot-scale RAS, each with a total volume of 1.7 m^3 (make-up water: 80 L/day) stocked with juvenile rainbow trout (*Oncorhynchus mykiss*). All systems had been operating with constant and identical feed loading of 3.13 kg feed/m^3 make-up water for a period of three months before the experiment was initiated. Three controlled levels of feed loading where established in duplicates: no feed (0 kg feed/m^3), unchanged feeding (3.13 kg feed/m^3), and doubled feeding (6.25 kg feed/m^3). The experimental period was seven weeks, where microbial and chemical water quality was monitored weekly. Bacterial activity was measured using Bactiquant®, and microbial hydrogen peroxide degradation. Bacterial abundance was quantified by flow cytometry, and water quality parameters by standardized methods.

The study showed that water quality as well as bacterial activity and abundance were affected by the changes in feed loading. The microbial water quality parameters, however, did not respond to feed loading changes as quickly and straightforward as the physicochemical parameters such as nitrate, chemical oxygen demand and biological oxygen demand. It was presumed that the fixed bed biofilter suppressed microbial response in the water phase. Hydrogen peroxide degradation assay proved to have considerable potential for assessing overall bacterial load in RAS water although further adjustments and standardization procedures are required.

1. INTRODUCTION

The aquatic environment in recirculating aquaculture systems (RAS) is complex, consisting of multiple biotic and abiotic water quality parameters (Timmons *et al*., 2009) including surface associated and suspended bacteria. Feed composition and digestibility (Lam *et al.*, 2008; Blancheton *et al.,* 2013), and feed loading (Pedersen *et al.*, 2012; von Ahnen *et al.*, 2015), significantly affect the aquatic environment and nutrient abundances in RAS. Dissolved compounds and fine particulate organic matter are complicated to remove, so micro particles accumulate within the system serving as substrate and surface area for heterotrophic bacteria (Wold *et al.*, 2013; Pedersen *et al.*, 2017). These bacteria-inhabited particles will degrade further until they eventually become part of the dissolved organic fraction of the water and sustain further bacterial growth. Several studies have shown that the bacterial population in RAS is highly dominated by heterotrophic bacteria (Leonard *et al.*, 2000; Michaud *et al.*, 2009; Michaud *et al.*, 2014; Rud *et al.*, 2017) both in suspension and on surfaces. These bacteria obtain energy from the degradation of organic carbon compounds (Prest *et al.*, 2016b). They occupy available niches that could potentially be used by specific pathogenic bacteria (Attramadal *et al.*, 2012; Blancheton *et al.*, 2013). However, high abundance of these bacteria may directly or indirectly affect the fish, acting as opportunistic pathogens or competing for oxygen and potentially affecting the nitrification process as they compete for space with the autotrophic bacteria (Blancheton *et al.*, 2013, Michaud *et al*., 2014).

In RAS, water quality parameters are controlled to maintain stable physicochemical water quality for the fish. Since bacteria are omnipresent in RAS, it is important to get a better understanding of the factors that cause changes in microbial water quality and, if possible, to understand how to regulate and control these factors in order to achieve biological stable RAS water of high quality. Monitoring tools are necessary to observe and control microbial water quality, but the available tools are generally complex or associated with a considerable time lag between water sampling and analysis results (Rojas-Tirado *et al.*, 2017). Moreover, no guidelines on which critical parameters to measure exist, and the range of acceptable levels and fluctuations are not known. Therefore, there is a need for new operational tools and for establishing associated guidelines to describe and control bacterial loads in RAS water.

To contribute to this, the following experiment was conducted to describe changes in microbial water quality in terms of bacterial activity and abundance associated with changes in feed loading. Six pilot scale RAS were operated under identical conditions for three months to reach steady state before changes in feed loading were made. Three groups with different feed loading were then established and concomitant changes in water quality parameters were monitored.

2. MATERIAL AND METHODS

2.1 System and experimental setup

The experiment was conducted in six identical, separate pilot scale RAS each operated under constant and identical conditions over a period of thirteen weeks prior to this study (Rojas-Tirado *et al.*, 2017). Following the thirteen week period of fixed feed loading (FL) (250 g feed/day per RAS and 80 L/day make-up water; corresponding to a feed loading of 3.13 kg/m^3 day), three different levels of feed loading were allocated to the six RAS (duplicate study): i) 0 kg/m^3 day (FL_0) ii) 3.13 kg/m^3 day ($FL_{3.13}$) and iii) 6.25 kg/m^3 day ($FL_{6.25}$) (Table 1). The effect of changed feed loading on bacterial activity and abundance was then evaluated for seven weeks (week 0 to week 7), where week 0 denotes the time of changed feed loading.

Details on system design and management can be found in Fig. 1 and Rojas-Tirado *et al.* (2017). Each RAS was stocked with 32.4 ± 0.49 kg rainbow trout (*Oncorhynchus mykiss*) and fed daily with commercial feed (EFICO Enviro 3 mm; Biomar, Denmark) from 9:00 to 15:00 by the use of a belt feeder. The photoperiod was from 7:30 to 22:00. Dissolved oxygen, temperature and pH were measured on a daily basis. Oxygen concentration was maintained above 80 % saturation, pH between 7.3-7.4 and, temperature 19 ± 0.3 °C.

Daily management of each RAS included: i) solids removal by emptying the accumulated feces in the sludge collectors at the bottom of the swirl separators (Fig. 1), ii) addition of 80 L make-up water (tap-water)/day per RAS (corresponding to 4.7 % of the system volume), iii) cleaning and loading of the belt feeders, and iv) addition of sodium bicarbonate equivalent to 20 % of the weight of the added feed to compensate alkalinity loss due to the nitrification process. The biofilters were not backwashed during the experimental period. Inspection and removal of any dead or moribund fish took place on a daily basis.

2.2 Water sampling and analysis

Grab samples of 2 L water were taken weekly from the outlet of the tanks (siphoned gently from the top of the swirl separators) of each RAS at 8:00 before feeding and management routines. The selected parameters used to assess the physicochemical and microbial water quality are described below and listed in Table 2 and 3, together with their sampling procedure, treatment and processing.

2.2.1 Physicochemical water quality parameters

Total and dissolved chemical oxygen demand (COD_{TOT} and COD_{DISS}) as well as total and dissolved biological oxygen demand ($BOD_{5\text{-}TOT}$ and $BOD_{5\text{-}DISS}$) were used to characterize the organic matter content in the water. The particulate fraction of COD and BOD (COD_{PART} and $BOD_{5\text{-}PART}$) were calculated by subtracting the dissolved fraction from the total (COD_{TOT} - COD_{DISS} = COD_{PART}; $BOD_{5\text{-}TOT}$ – $BOD_{5\text{-}DISS}$ = $BOD_{5\text{-}PART}$). Additional analyses including total ammonia nitrogen (TAN), nitrite-nitrogen (NO_2^--N) and nitrate-nitrogen (NO_3^--N) were performed on filtered samples stored at 4 °C until analysis. Table 2 specifies the sampling procedure, and treatment and analysis of the water samples for each of the physicochemical water quality parameters.

The submerged, fixed-bed biofilters were not backwashed during the experiment but at the end of the experiment (week 7) organic matter accumulated at the bottom was assessed. Six hours after stopping the pumps and subsequent settling of solids in the biofilters, 80 L were collected from a bottom drain of each

biofilter. These six collections were homogenized and 2 L subsamples from each were used for analysis of total solids (TS) and ashes.

2.2.2 Microbial water quality assessment

Bacterial activity

Bacterial activity in the water phase was assessed by two different methods, BactiQuant® (Mycometer, Hillerød, Denmark) and hydrogen peroxide degradation assay. Bactiquant measures bacterial activity indirectly via a common hydrolase enzyme found within a wide range of bacteria (Reeslev *et al.*, 2011). A well-defined water volume was filtered through a 0.22 μm filter, on which particle-bound and free bacteria are trapped; the filter cake is then exposed to a fluorescent substrate and depending on the amount of bacteria present and their activity, a quantitative fluorescent signal can be detected. The BQ values (BQV) were calculated according the sample volume (10 ml), exposure time (30 min) and incubation temperature (measured on site) as described by Rojas-Tirado *et al.*, (2017) and Pedersen *et al.* (2017).

The hydrogen peroxide (HP) degradation assay applied was derived from the principle of microbial activity degradation kinetics described in Arvin and Pedersen (2015). The method quantifies the sum of enzymatic degradation of HP (Hossetti and Frost, 1994) in a water sample based on the presence and activity of free and particle-bound bacteria. A high bacterial activity and abundance in the water phase causes a fast HP degradation where more than 20 mg HP/l can be degraded in less than one hour (Pedersen, 2013). The degradation kinetics can be described as a first order reaction by the exponential decay equation: $C_t = C_0 \cdot e^{-kt}$, with k being the descriptive reaction rate constant (per hour), C_0 the initial concentration of HP (mg/L), C_t the concentration at time "t" in hours (h). Water samples were taken from the outlet of the tank from each RAS and transferred to 500 ml beakers, stirred at 250 RPM at room temperature. Beakers were then spiked with HP to reach an initial nominal concentration of 8 mg HP/L. Hydrogen peroxide concentrations were measured 2, 10, 30 and 60 minutes after HP addition by the spectrophotometric method described by Tanner and Wong (1998) and modified by Pedersen and Pedersen (2012). Hydrogen peroxide degradation was measured on water samples from each system for three consecutive weeks towards the end of the experiment.

Bacterial abundance assessment

The total number of bacterial cells was quantified by flow cytometry using a BD Accuri™ C6 Flow Cytometer (BD Bioscience, San Jose, CA, USA), using staining of DNA with SYBR Green I (nucleic-acid gel stain, Molecular Probes Invitrogen) and excitation with the blue laser (488 nm) (Marie *et al.*, 2005; Wold *et al.*, 2014). The threshold for the FL1-A channel was set at $10^{3.25}$. Signals above that threshold were considered as bacterial cells, and signals below were considered to be background signals. Water samples did not receive any treatment that could provide cell detachment from particles, so the data obtained can be regarded as bacteria cells suspended in the water phase ("free-living bacteria"). Water samples were checked for presence of phytoplankton, but none were detected.

2.3 Assessment of fish performance

Fish biomass in each system was measured five weeks (week -5) before changes in feed loading and at the end of the experiment (week 7). Feed conversion ratio (FCR; feed intake/biomass gain) and specific growth rate (SGR) were calculated according to Hopkins (1992).

2.4 Data analysis

The different parameters measured are presented as mean ± standard deviation. Data were log-transformed when necessary to meet normality (normal distribution). One-way analysis of variance (ANOVA) was applied to test for difference between treatments at week 0 and week 7. For data not meeting the homoscedasticity assumption, the one-way ANOVA on ranks (Kruskal-Wallis) was performed. Difference in treatment means were tested by Tukey´s least square means test, with a significance level set at $p < 0.05$. Statistics were performed using the software SigmaPlot 12.5 from Systat Software, Inc., San Jose California USA.

3. RESULTS

3.1 Dissolved N and organic matter

TAN and nitrite remained stable at low concentrations throughout the experiment in all six RAS (Table 4). Nitrate-N concentrations immediately started to diverge when feed loadings were changed (from week 1; Fig. 2). Nitrate concentrations decreased from 133 ± 1 mg NO_3-N/L to 59 ± 0 mg NO_3-N/L in the water from the FL_0 RAS, stayed constant for the $FL_{3.13}$ (133 ± 0.9 to 159 ± 1.3 mg NO_3-N/L), and increased steadily in the water from the $FL_{6.25}$ RAS (133 ± 5.6 to 280 ± 11 mg NO_3-N/L).

The biodegradable organic matter ($BOD_{5\text{-}TOT}$) was significantly reduced by 55 % at week 7 in FL_0 (Fig. 3; $p < 0.05$). The $BOD_{5\text{-}TOT}$ in the unchanged RAS ($FL_{3.13}$) increased somewhat, peaking at 9.5 ± 1.6 mg O_2/L in week 5 and ending at 6.3 ± 2.84 in week 7. In comparison, transient levels up to 20 mg O_2/L were observed in the $FL_{6.25}$ RAS, 5-6 weeks after feeding was doubled and ended up at 7.19 ± 1.6 mg O_2/L (Fig. 3). The dissolved fraction of BOD in the FL_0 RAS was reduced by approximately 86 % at the end of the experiment (from 2.93 ± 0.22 to 0.4 ± 0.13 mg O_2/L; Table 4), which is low compared to the other two treatments (3.3 ± 0.8 and 5.8 ± 1.94 mg O_2/L in the $FL_{3.13}$ and $FL_{6.25}$ RAS, respectively; Table 4). Decrease in dissolved BOD in the FL_0 RAS correlated significantly ($r = 0.75$; $p < 0.05$; $n = 14$) with NO_3-N concentration, which decreased due to dilution.

Total COD decreased by 33 % in the FL_0 RAS ending at 34.0 ± 6.3 mg O_2/L, whereas the unchanged RAS ($FL_{3.13}$) and the $FL_{6.25}$ RAS increased with 16 and 5 %, respectively (ended at 65.3 ± 18.7 and 82.2 ± 7.8 mg O_2/L; Table 4). The dissolved COD fraction (COD_{DISS}) in FL_0 RAS steadily decreased from 37 ± 2 mg O_2/L to 16 ± 1 mg O_2/L (Fig. 4; Table 4), ending significantly lower ($p < 0.05$) than the other two treatment groups (33 ± 2 and 45 ± 5 mg O_2/l, respectively). The decrease in COD_{DISS} in the FL_0 RAS was highly correlated to the reduction in NO_3-N ($r = 0.97$; $p < 0.0001$). The particulate COD fraction (COD_{PART}) increased transiently by 4-6 fold in the FL_0 RAS, reaching levels above 40 and 60 mg O_2/L in week 3-5 in the two unfed RAS (Fig. 4, a). In week 7, at the end of the experiment, it was 18.25 ± 5.1 mg O_2/L compared to 32.8 ± 17.2 and 38.0 ± 2.44 mg O_2/L in $FL_{3.13}$ and $FL_{6.25}$ RAS, respectively (Table 4).

The $BOD_{5\text{-}TOT}$: COD_{TOT} ratio changed from 1:8 to 1:16 in FL_0, whereas in the other two treatment groups it remained stable around 1:11. The biodegradability index: BOD_5/COD (Srinivas, 2008) was thus around 0.1 in all RAS during the trial, except for the FL_0 RAS ending at 0.06.

Dry matter content in the reject water from the standardized biofilter backwash performed at the end of the experiment (week 7) was positive and significantly related ($p < 0.05$) to feed loading with 1.5, 16 and 32 g/L for FL_0, $FL_{3.13}$, and $FL_{6.25}$ RAS, respectively. The associated ash content followed the same pattern with 0.7 g/L, 4 g/L, and 8 g/L in FL_0, $FL_{3.13}$ and $FL_{6.25}$ RAS, respectively.

3.2 Microbial water quality parameters

3.2.1 Bacterial activity assessments

Bacterial activity measured by Bactiquant®, showed that the six RAS had activities in the range of 3-9 $\times 10^4$ BQV/ml before changing the feed loadings (Fig. 5). At the end of the experiment, the bacterial activity in the FL_0 RAS (5.2-5.6 $\times 10^4$ BQV/ml) was 2-4 times lower ($p < 0.05$) than in the $FL_{3.13}$ and the $FL_{6.25}$ RAS's (1-1.5 $\times 10^5$ and 1.1-1.3 $\times 10^5$ BQV/ml, respectively).

Hydrogen peroxide (HP) degradation rates in the water from all RAS were significantly affected by feed loading ($p < 0.001$). The lowest HP removal rate was measured in the water from the unfed RAS, FL_0 (1.4 ± 0.3 mg/L reduction in HP concentration after 30 min) with a mean rate constant (*k*) of 0.41 h^{-1} (Fig. 6, a). The water from the $FL_{3.13}$ RAS had a 4 ± 0.36 mg/L reduction in HP concentration after 30 minutes (Fig. 6, b) and a mean rate constant of 1.73 ± 0.38 h^{-1}, whereas almost complete removal of HP (6 ± 0.49 mg HP/L reduction within 30 min) was observed in the water from the $FL_{6.25}$ RAS (Fig. 6, c) reaching a mean rate constant of 4.92 ± 0.86 h^{-1} at the end of the trial.

3.2.2 Bacterial abundance

Flow cytometry showed that the concentration of free-living bacteria in the six RAS was in the range of 0.6 – 3.8 $\times 10^7$ cells/ml before changing the feed loadings. The abundance decreased from 2.0-2.4 $\times 10^7$ to 2.7-4.7 $\times 10^6$ cells/ml in the FL_0 RAS (Fig. 7a) towards the end of the experiment (week 7). The number of free-living bacteria in the $FL_{3.13}$ RAS was stable (0.8-1.2 $\times 10^7$ cells/ml in week 7; Fig. 7, b), whereas in $FL_{6.25}$ an increase was observed over time (ending at 5.3-9.5 $\times 10^7$ cells/ml; Fig. 7, c). The replicated RAS systems behaved fairly similar, but deviations increased with increasing feed loadings.

3.3 Fish performance

Fish biomass in the $FL_{3.13}$ and $FL_{6.25}$ RAS increased by 14 and 28 kg/RAS, respectively, during the experiment, reaching 47 ± 0.49 and 61 ± 1.72 kg/RAS, respectively. The fish biomass in the FL_0 RAS was reduced by 0.4 kg/RAS. The FCR was 1.34 and 1.12 and SGR 0.48 and 0.81 % in the $FL_{3.13}$ and $FL_{6.25}$ RAS, respectively (FCR and SGR in the FL_0 RAS not considered). Only limited fish mortality was observed over the 10 weeks experimental period, in total ranging between 1 and 4 % of total biomass. The mortality in FL_0 was slightly higher than in $FL_{3.13}$ and $FL_{6.25}$, however not significant.

4. DISCUSSION

4.1 Physicochemical water quality assessment

The feed composition and feed loading applied and the concomitant TAN and urea excretion from the fish (Dalsgaard *et al.*, 2015) dictates the production of nitrate in RAS with well-functioning biofilters. Nitrate concentrations are hence predictable in RAS under steady state conditions, provided no removal of nitrate by denitrification (Colt *et al.*, 2006; Eding *et al.*, 2006; Pedersen *et al.*, 2012). Accordingly, three distinct scenarios in development of nitrate concentration were observed after the changes in feed loading (Fig. 2). The nitrate levels for the FL_0 RAS decreased exponentially towards 0 mg NO_3-N/L due to more-or-less ceased nitrate production and ongoing dilution. In the $FL_{6.25}$ RAS, nitrate increased towards twice the concentration of the unchanged RAS ($FL_{3.13}$) but did not reach a plateau twice the value of the NO_3-N of $FL_{3.13}$, which would have indicated a new steady state condition for $FL_{6.25}$. The steady, minuscule increase in nitrate concentration in $FL_{3.13}$ throughout the 10 weeks periods possibly reflects the slight increases in FCR (and increased TAN excretion) associated with fish getting larger, or it might be the asymptotic approach to complete balance.

The sudden increase in feed loading in $FL_{6.25}$ did not cause any marked increase in TAN or nitrite concentrations, presumably due to the maturity of the system, the capacity of the biofilter applied and to substrate-dependent nitrification kinetics (Pedersen *et al.*, 2015; von Ahnen *et al.*, 2015). A doubling of ammonium loading is not a problem for a mature biofilm as long as it does not go into oxygen limitation (Harremoës and Henze, 1997).

The organic matter concentrations showed similar but less distinct patterns compared to the changes in nitrate concentrations. Before altered feed loadings $BOD_{5\text{-}TOT}$ levels ranged between 5 and 10 mg O_2/L, even though all 6 RAS were kept under constant and identical conditions. This emphasizes the fact that identical RAS may differ substantially in some water quality parameters that actually affects bacterial communities. Whether such variation is unavoidable in biological systems or can be pinpointed to specific reasons cannot be determined from this study, but hydraulic conditions in the biofilter, uneven entrapment or liberation of particulate organic matter from the biofilter compartment (Fernandes *et al.*, 2017) and/or predation by protozoa and metazoa may affect systems specific carbon balances. Despite the initial variation, $BOD_{5\text{-}TOT}$ in all systems diverged in accordance with changes in feed loading.

Likewise, the dissolved COD in FL_0 also decreased right after the feeding was ceased, while it remained relatively stable in the other two treatment groups. The decrease in COD_{DISS} in the FL_0 RAS was strongly correlated to the dilution. On the other hand, only a minor increase in COD_{DISS} was observed in the $FL_{6.25}$ RAS, indicating a balance between production and removal of COD_{DISS} despite the loading.

The particulate fraction (COD_{PART}), however, did not respond in any linear or straightforward way to the feed loading because the largest increases and fluctuations were observed in the FL_0 RAS. This abrupt increment was not reflected in any BOD-fraction (data not shown), strongly suggesting that this transient increase in COD_{PART} was caused by biofilm release as a consequence of stopped feeding. For the $FL_{3.13}$ and $FL_{6.25}$ RAS, the COD_{PART} remained more stable, although some increase and also variation was observed between the $FL_{3.13}$ systems at the end of the experiment. The COD_{DISS}/COD_{PART} ratio in $FL_{3.13}$ at week 7 was 0.99, which is in accordance with Fernandes *et al.* (2015) who found a ratio of 0.93 after 19 weeks of operation under similar

conditions. Probably more interesting, the difference between the FL groups was even more pronounced when comparing $BOD_{5\text{-}DISS}/BOD_{5\text{-}PART}$. Ratios of 0.23, 1.14, and 4.14 were observed for FL_0, $FL_{3.13}$ and $FL_{6.25}$, respectively, suggesting that this ratio could be used as a tool to indicate relative differences in feed loading and water quality between systems. The biodegradability index ($BOD_{5\text{-}TOT}/COD_{TOT}$) in the $FL_{3.13}$ and $FL_{6.25}$ RAS was approximately 0.09 at the end of the experiment, implying that the organic matter accumulating within the systems was > 90% recalcitrant (Rojas-Tirado *et al.*, 2017). In the FL_0 RAS the biodegradability index dropped to 0.06 at the end of the experiment, indicating a faster reduction in BOD than in COD, as could be expected.

The sludge collected from the fixed bed biofilters at the backwash event in week 7 was positively related to the feed loading level. The FL_0 RAS accumulated only 10% of the amount of sludge in the $FL_{3.13}$ RAS. The $FL_{6.25}$ RAS accumulated twice the sludge of the $FL_{3.13}$ RAS. How deposition of particulate organic matter in fixed bed biofilters affects fluxes of dissolved and particulate organic matter as well as interactions between decomposers and grazers deserves future attention.

4.2 Microbial water quality assessment

Bacterial activity

Bactiquant® levels were positively correlated to the changes in feed loading although a certain delay in response was observed. This is different from the immediate changes that occurred in nitrate concentrations. Bacterial activity in the FL_0 RAS did not decline after the feeding was stopped, but increased slightly towards the end of the experiment. Bactiquant® assesses bacterial activity by measuring a specific hydrolase enzyme found in most of bacteria, and Pedersen *et al.* (2017) demonstrated a linear correlation of Bactiquant® activity to the available surface area of particulate organic matter in less intense RAS water. The constant bacterial activity levels in FL_0 RAS suggest that the accumulation of particulate waste during the initial operation (before stopped feeding) was sufficient to sustain the bacterial growth on particles. This is supported by the fact that COD_{PART} actually increased in FL_0. The $FL_{3.13}$ RAS - not subjected to changes – had increasing BQV in both RAS units, indicating that the systems at week 0 were not in steady state from a bacterial activity point of view. The Bactiquant®-response was related to the increases in organic matter levels observed ($BOD_{5\text{-}TOT}$ and COD_{TOT}) in $FL_{3.13}$ and in $FL_{6.25}$ in particular. The BQV levels observed, range 2.7 $\times 10^4$ - 1.5 $\times 10^5$ BQV/ml, fit well to levels found in intensive RAS (Pedersen *et al.*, 2017). Bacterial activity in the $FL_{6.25}$ systems, showed almost identical patterns between RAS duplicates, with a 3-4 weeks delay before a significant increase was observed. The increase might be expected as a result of the doubling in the feed load and the corresponding increase in waste excretion. However, apparently the biofilter was able to attenuate this change for a period of time.

Bacterial activity assessed by hydrogen peroxide degradation assay was significantly related to the feed loading ($p < 0.001$), supporting the hypothesis that feed loading directly dictates available organic matter influencing the microbial abundance and activity in RAS waters. Water from the FL_0 RAS had a significant removal of HP even 4-6 weeks after termination of feeding, suggesting a prolonged contribution of organic matter from e.g. biofilm release or from sludge, deposited in the biofilter. Arvin and Pedersen (2015) showed that HP degradation is a biotic process (no degradation of HP in autoclaved RAS water), related to microbial enzymatic activity rather than potential degradation due to inorganic catalysts (Pardieck *et al.*, 1992). The HP degradation assay applied turned out to be predictive and with sufficient reproducibility, pending to be

implemented as a new, simple and fast method to evaluate bacterial water quality. Since HP degradation is dependent on temperature and to a nominal concentration, assays have to be performed under similar conditions in order to compare different water matrices and thus standard procedures has to be developed for the method to be universally applied.

Bacterial abundance of free-living cells

Cell counting using flow cytometry have not been widely used to assess RAS microbial water quality, but some studies related to rearing of marine larvae in RAS have reported densities of 6-8 $\times$ 10^6 cell/ml and 1 $\times$ 10^7 cell/ml (Attramadal *et al.*, 2012, 2014), and 2 $\times$ 10^6 cell/ml (Wold *et al.*, 2014), 2 $\times$ 10^7 cell/ml in rearing water, and 0.1-6 $\times$ 10^6 cell/ml in pure seawater inlet (van der Meeren *et al.*, 2011). Drinking water ranges between 10^3 to 10^6 cells/ml (Prest *et al.*, 2016a) and in this trial tap water used to fill the systems contained 0.5-1.4 $\times$ 10^6 cells/ml. These data may not be directly comparable to this study due to large differences in experimental setups, especially feed loading. However, the cell numbers obtained in the present study are within the same range.

The free-living cells in RAS water showed a direct response to changes in feed loading. The abundance in FL_0 RAS declined immediately after feed stop associated to dilution of the systems and the concomitant decline in dissolved, readily available organic matter ($BOD_{5\text{-}DISS}$). However, a considerable amount of cells (2.7 – 4.7 $\times$ 10^6 cells/ml) were still measured at the end of the experiment for the FL_0 RAS. Although no distinction between dead and living cells were made, the bacterial activity assessed by Bactiquant® supports the presence of active cells. As discussed above, bacterial activity expressed by BQV may have slightly overrated bacteria attached to particles since they can have higher extracellular enzymatic activity per cell than free-living bacteria (Karner and Herndl, 1992; Smith *et al.*, 1995). The activity of the particle- associated bacteria, and microbial degradation of organic matter in the biofilter as well as dead microbial biomass, could have provided substrate for the free-living bacteria in the dissolved-substrate limited water of the FL_0 RAS. This interaction should also be expected to happen in the other treatments as well, but in a more dynamic way. The $FL_{3.13}$ RAS showed a baseline of 0.5 – 4 $\times$ 10^7 cell/ml between week 0 to week 7, ending with 60% more free-living bacteria than the FL_0 RAS. The $FL_{6.25}$ RAS had a comparable and simultaneous development with positive response and a pronounced increase in numbers at the end of the trial, despite some difference in cell concentration between duplicates, exceeding the $FL_{3.13}$ RAS by five to nine times in cell number at the end of the experiment. The final free-living cell concentrations within the treatments were consistent with the final values of the $BOD_{5\text{-}DISS}$. The $FL_{6.25}$ RAS had a 41% higher concentration of available organic carbon (reflected in the $BOD_{5\text{-}DISS}$) for further growth compared to the unchanged RAS ($FL_{3.13}$).

4.3 Implications and challenges in RAS microbial water quality

Increased feed loading caused direct but somewhat delayed responses in terms of bacterial activity and abundance, implying that probably the biofilter attenuated the bacterial response in the water phase when feed loading was increased. Mature biofilms shows rapid response to increased loading of inorganic and organic nutrients to the system, and may also contribute to the dispersion of new bacterial cells into the water (Leonard *et al.*, 2000; Davies, 2011; McDougald *et al.*, 2011). In this study, the results showed an almost immediate and constant increase in numbers of free-living bacteria in the $FL_{6.25}$ RAS without manipulating

the C/N ratio (Leonard *et al.*, 2002; Michaud *et al.*, 2006). It seems that biologically mature biofilter systems have the potential to assimilate a sudden increase in feed loading with concomitant increase in attached and free-living bacteria. The difference in response between free-living bacteria and particle-associated bacteria could have been related to the capacity of fixed bed biofilter for micro particles entrapment (Fernandes *et al.*, 2017). As mentioned before, Bactiquant® is highly associated to bacteria attached to particles, and the transition of water passing through the biofilter could have suppressed the response in bacterial activity response in the water phase.

Heterotrophic and autotrophic bacteria will be in balance at system level as long the C/N ratio is not dramatically changed by e.g. excess feed waste or insufficient solids removal (Fernandes *et al.*, 2015) and the autotrophs did not suffer from oxygen limitation. To evaluate such changes or to quantify effects of disinfection (Attramadal *et al.*, 2014), new measures to detect bacterial abundance and activity including live/dead assays are needed.

A number of well-known factors (e.g. organic and inorganic nutrients, temperature, pH, and predation) regulate bacterial growth in water (Blancheton *et al.*, 2013; Gerardi, 2006; Rurangwa and Verdegem, 2015; Prest *et al.*, 2016a), and more knowledge is needed to fully understand the interaction of these factors in RAS. Monitoring tools like Bactiquant®, HP degradation assay, flow cytometry - used in this study, and others like online flow cytometry (Besmer *et al.*, 2014), Bacmon (Grundfos A.S, Højris *et al.*, 2016) and ATPase assay (Vang *et al.*, 2014) are all new measures that might provide means for an increased understanding of the microbial dynamics within RAS. These approaches may all contribute to improving our understanding of the complex microbial interactions in RAS in future studies. Additionally, surveys on full-scale RAS may also increase our knowledge on how various factors affects the bacterial dynamics within a system that is constantly challenged by variations in nutrient and organic loading.

5. CONCLUSION

This study demonstrated the dynamics in microbial water quality parameters as a function of increased and decreased feed loading in a set of controlled pilot-scale RAS using two new fast and practical assays and flow cytometry. The main conclusions are:

- Changes in feed loading caused substantial effects on selected chemical and microbial water quality parameters. As the chemical response is immediate, microbial water quality response speed is dependent on other influencing factors within RAS.
- Submerged biofilters attenuated the microbial response and fluctuations in microbial water quality in RAS water when feed loading was increased.
- Bacterial activity measured as BQV or as HP degradation rate, responded to altered feed loading after some weeks.
- Free-living bacteria responded to changes in the concentrations of dissolved organic matter.
- Bacterial activity and abundance within mature RAS were affected by substrate availability, solids removal and particulate matter (surface area) and the submerged fixed-bed biofilter attenuated the response observed in the water phase.

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Figures

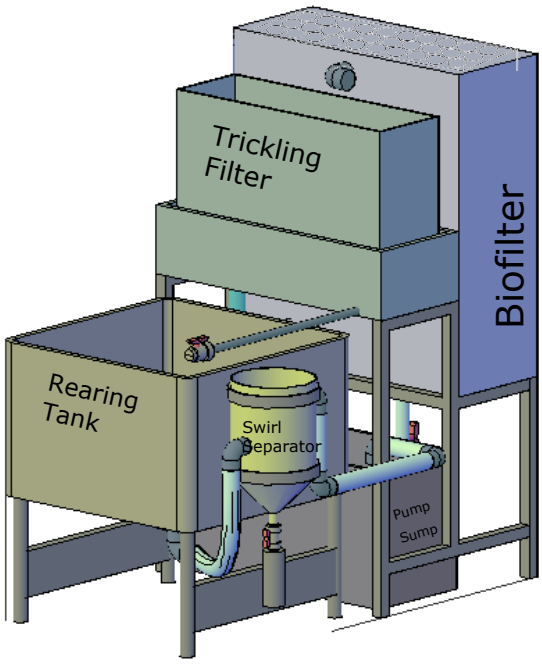

Fig. 1: Scheme of the RAS configuration, consisting of a fixed-bed biofilter (0.76 m^3), trickling filter, rearing tank, swirl separator, and pump sump.

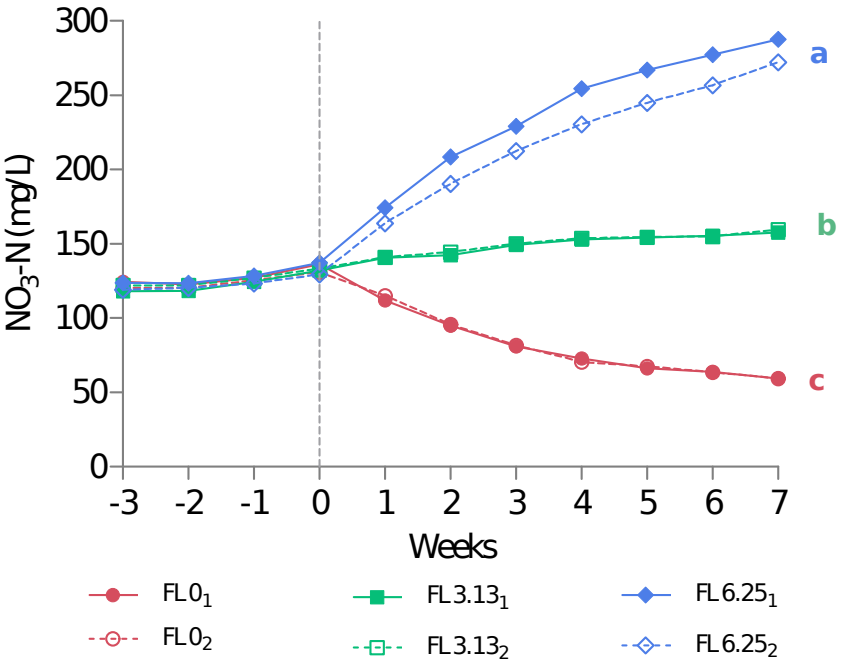

Fig. 2: Nitrate concentration over a period of 10 weeks for the different treatments and RAS duplicates: 0 kg feed/m^3 (FL_0); 3.13 kg feed/m^3 ($FL_{3.13}$); and 6.25 kg feed/m^3 ($FL_{6.25}$). Week 0 and the vertical line indicates the last measurement just before changes were made in feed loading. The minus weeks indicates concentration of nitrate in RAS water before changes. Different superscript indicates statistical difference at week 7.

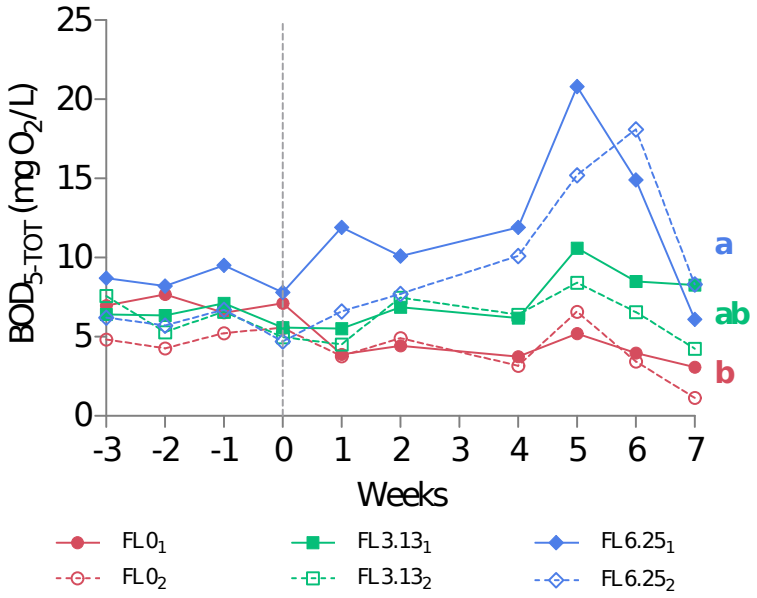

Fig. 3: Total biological oxygen demand ($BOD_{5\text{-}TOT}$) concentration over a period of 10 weeks for the different treatments and RAS duplicates: 0 kg feed/m³ (FL_0); 3.13 kg feed/m³ ($FL_{3.13}$); and 6.25 kg feed/m³ ($FL_{6.25}$). Week 0 and the vertical line indicates the last measurement before changes in feed loading were made. The minus weeks indicates concentration of $BOD_{5\text{-}TOT}$ in RAS water before changes. Different superscript indicates statistical difference at week 7.

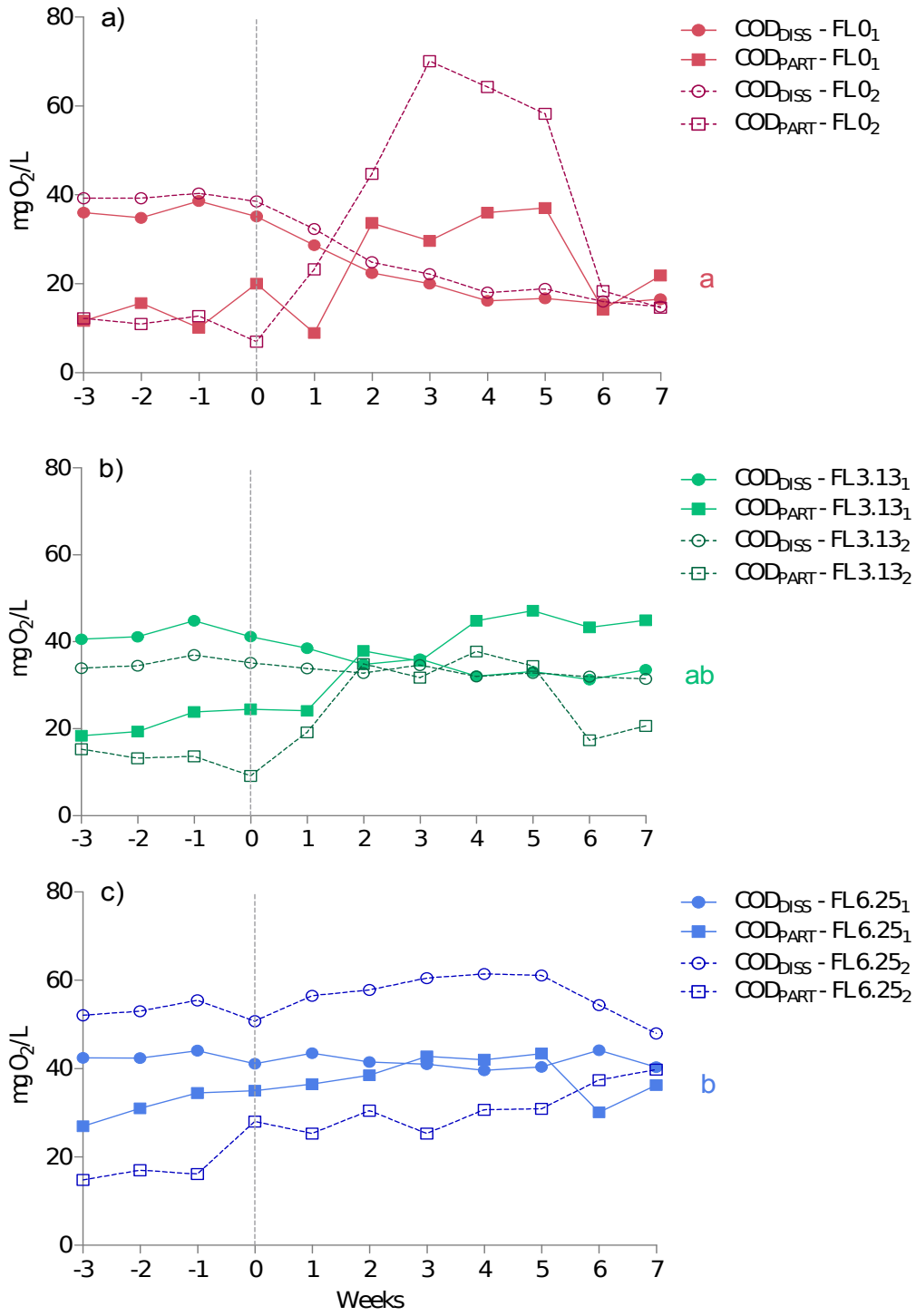

Fig. 4: Concentration of the dissolved and particulate fractions of COD over a period of 10 weeks for the different treatments and RAS duplicates: 0 kg feed/m³ (FL_0); 3.13 kg feed/m³ ($FL_{3.13}$); and 6.25 kg feed/m³ ($FL_{6.25}$). Week 0 and the vertical line indicates the last measurement before changes were made in feed loading. The minus weeks indicates concentration COD_{DISS} and COD_{PART} in RAS water before changes. Different superscript indicates statistical difference for COD_{DISS} between treatments at week 7.

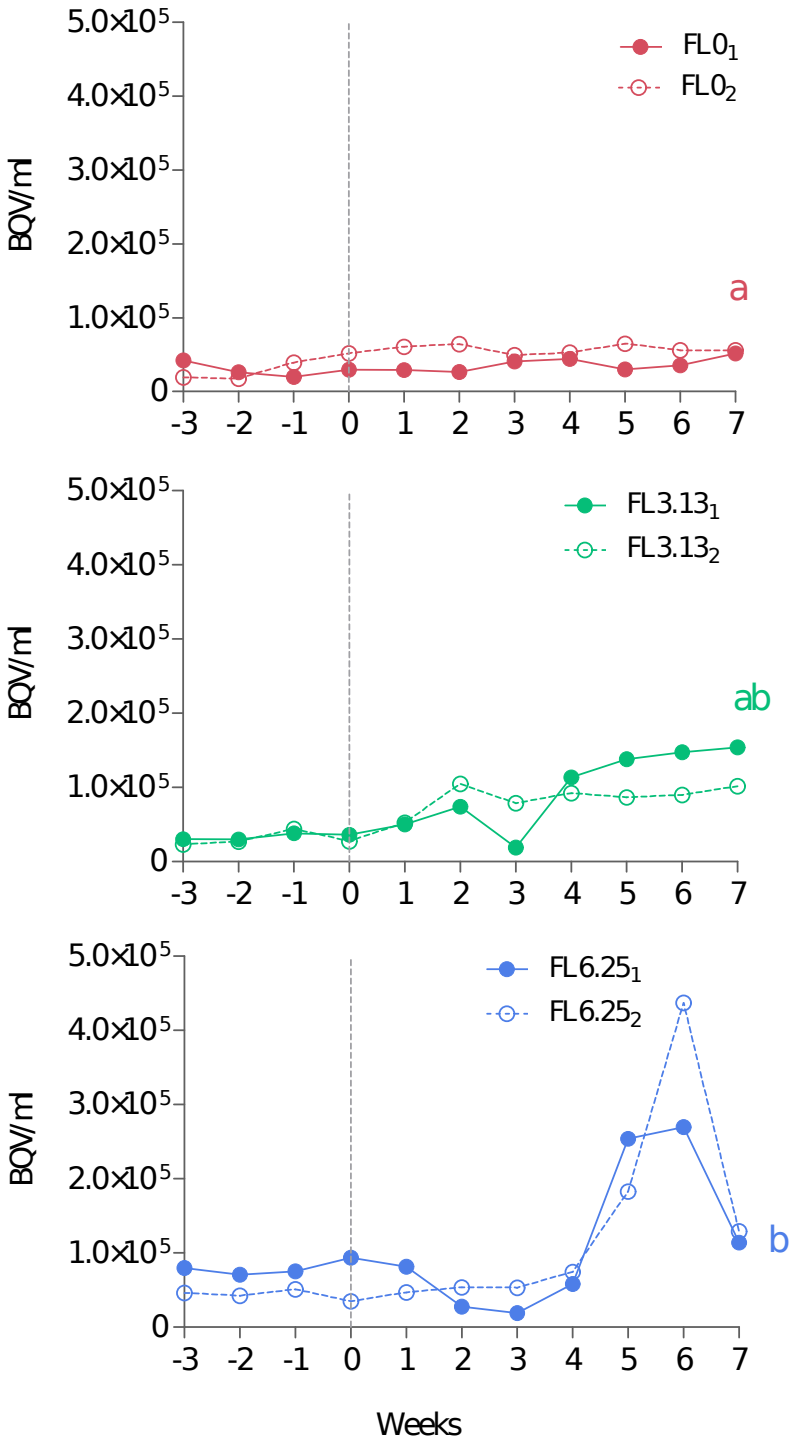

Fig. 5: Bacterial activity measured as bactiquant values (BQV) over a period of 10 weeks for the different treatments and RAS duplicates: 0 kg feed/m³ (FL_0); 3.13 kg feed/m³ ($FL_{3.13}$); and 6.25 kg feed/m³ ($FL_{6.25}$). Week 0 and the vertical line indicates the last measurement before changes were made in feed loading. The minus weeks indicates concentration of BQV in RAS water before changes. Different superscript indicates statistical difference between treatments at week 7.

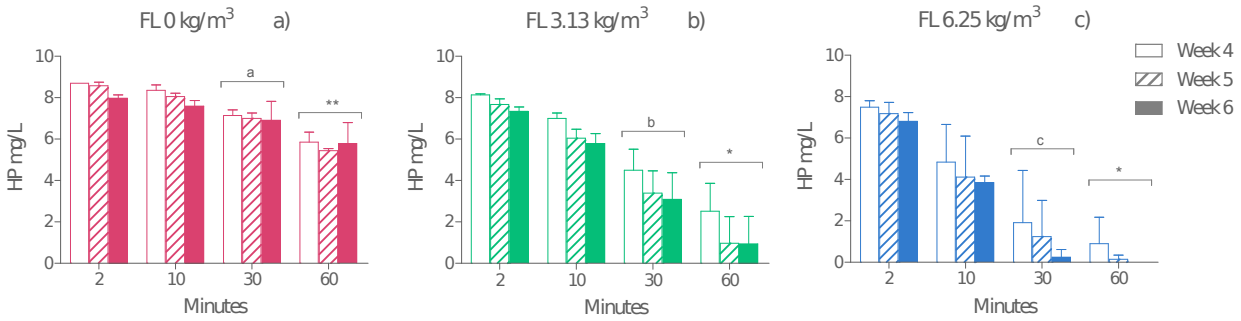

Fig. 6: Hydrogen peroxide concentration (mean ± SD, n=2) measured during 60 minutes in water samples from three different feed loadings (FL): a) FL 0 kg/m³; b) FL 3.13 kg/m³; and c) 6.25 kg/m³. Test was performed for water samples in week 4, 5 and 6. Blank bars: week 4; striped bars: week 5; and grey bars: week 6. Different superscript (30 min) and asterisk (60 min) indicates statistical difference between treatments (Tukey´s test; α = 0.05). Removal rate constants (*k*) achieved were (mean ± SD, n=3): a) 0.41 ± 0.05/h for FL 0 kg/m³; b) 1.73 ± 0.38/h for FL 3.13 kg/m³; and c) 4.92 ± 0.86/h for FL 6.25 kg/m³.

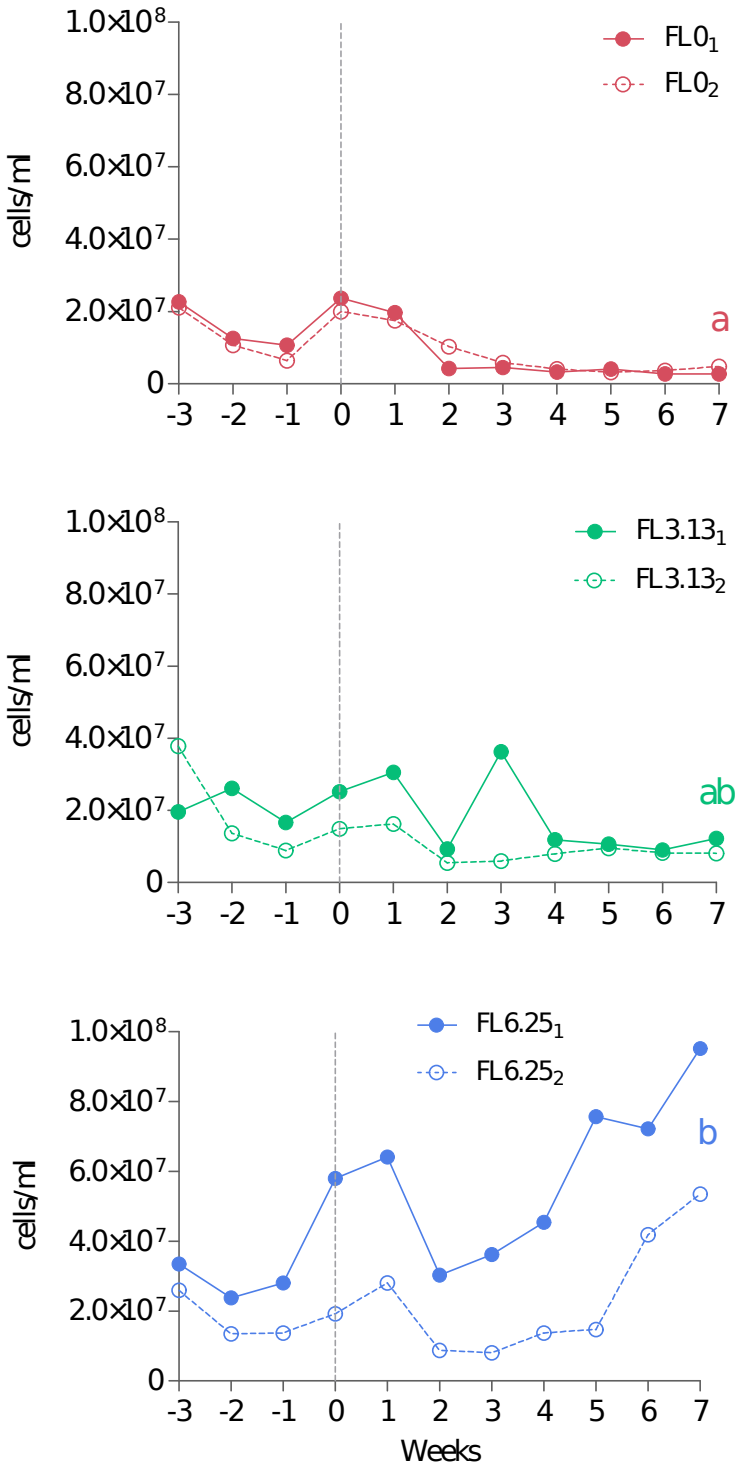

Fig. 7: Concentration of free-living bacteria in RAS water from three different feed loadings: a) 0 kg feed/m³ (FL_0); b) 3.13 kg feed/m³ ($FL_{3.13}$); and c) 6.25 kg feed/m³ ($FL_{6.25}$). All graphs shown with time line of 10 weeks. Week 0 and the vertical line indicates the last measurement before changes in feed loading were made. The minus weeks indicates concentration of bacterial cells in RAS water before changes in feed loading were made. Different superscript indicates statistical difference between treatments (Tukey´s test; $\alpha = 0.05$).

Tables

Table 1: Feeding load for maturation period and three treatment groups.

	Maturation period (Three months)	Treatment Groups		
		FL_0	$FL_{3.13}$	$FL_{6.25}$
Feed quantity (g/d)	250	0	250	500
Water exchanged per day (m^3/d)	0.08	0.08	0.08	0.08
Water renewal rate (m^3/kg feed)	0.32	-	0.32	0.16
Feed loading (kg/m^3)	**3.13**	**0**	**3.13**	**6.25**

Table 2: Chemical water quality parameters and analytical methods applied.

Parameter	Abbreviation	Units	Sample treatment and processing	Analytical Method	Reference	Frequency of Measurement
Temperature, pH, dissolved oxygen	Temp., pH, O_2	°C , pH units, mg/L	Direct / on location	Hach HQ40d instrument, Hach Lange, Germany	N/A	Daily
Chemical oxygen demand	COD_{TOT}	mg O_2/L	Unfiltered + acid addition and kept at 4°C.	LCK 914, Hach Lange, Germany	N/A	Weekly
Dissolved chemical oxygen demand	COD_{DISS}	mg O_2/L	Filtered 0.22 µm and kept at 4°C.	LCK 914, Hach Lange, Germany	N/A	Weekly
Particulate chemical oxygen demand	COD_{PART}	mg O_2/L	N/A	$COD_{PART} = COD_{TOT} - COD_{DISS}$	N/A	Weekly
Biochemical oxygen demand within 5 days	$BOD_{5\text{-}TOT}$	mg O_2/L	Unfiltered	Potientiometry/O_2 probe (WTW Oxi 340i)	ISO 5815	Weekly
Dissolved biochemical oxygen demand within 5 days	$BOD_{5\text{-}DISS}$	mg O_2/L	Filtered 1.6 µm	Potientiometry/O_2 probe (WTW Oxi 340i)	ISO 5815	Weekly
Particulate biochemical oxygen demand within 5 days	$BOD_{5\text{-}PART}$	mg O_2/L	N/A	$BOD_{5\text{-}PART} = BOD_{5\text{-}TOT} - BOD_{5\text{-}DISS}$	N/A	Weekly
Total solids (dry matter) and ashes	-	g/L	Unfiltered	Gravimetric	NMKL.23	End of trial
Total ammonia nitrogen	TAN	mg/L	Filtered 0.22 µm. Conserved at 4°C.	Colorimetry	DS 224	Weekly
Nitrite	NO_2-N	mg/L	Filtered 0.22 µm. Conserved at 4°C.	Colorimetry	DS 223	Weekly
Nitrate	NO_3-N	mg/L	Filtered 0.22 µm. Conserved at 4°C.	Colorimetry	ISO 7890/1	Weekly

Table 3: Microbial water quality parameters and analytical methods applied.

Parameter	Abbreviation	Units	Sample treatment and processing	Analytical method	Reference	Frequency of measurement
Bacterial Activity	BactiQuant Value	BQV	Unfiltered. Processed immediately	BactiQuant® (Mycometer, Denmark)	Manufacturers protocol	Weekly
HP degradation	HP	HP mg/L or h^{-1}	Unfiltered. Processed immediately	Colorimetry	(Arvin and Pedersen, 2015)	Week 4, 5 and 6
Bacteria cell number	Cell number	cell/ml	Unfiltered. Fixed with glutaric aldehyde (1% final concentration). Freezed immediately with liquid nitrogen gas and conserved at -20°C. Processed 6 months later.	Stained with Sybr Green I and counted with Flow Cytometer (Becton Dickinson FACscan)	(Marie *et al.*, 2005; Wold *et al.*, 2014)	Weekly

Table 4: Water quality parameters before changes in feed loading were made (week 0) and at the end of experiment (week 7) for each treatment.

Parameters	Units	Week 0			Week 7		
		FL_0 (n = 2)	$FL_{3.13}$ (n = 2)	$FL_{6.25}$ (n = 2)	FL_0 (n = 2)	$FL_{3.13}$ (n = 2)	$FL_{6.25}$ (n = 2)
Dissolved Oxygen	mg/L	8.2 ± 0.01	9.1 ± 0.48	8.6 ± 1.21	8.3 ± 0.19	7.9 ± 0.68	6.9 ± 1.27
pH		7.3 ± 0.04	7.4 ± 0.01	7.3 ± 0.03	7.3 ± 0.08	7.3 ± 0	7.4 ± 0.22
Temperature	°C	19.4 ± 0.14	19.1 ± 0.21	19.7 ± 0.07	18.8 ± 0.07	19.1 ± 0.07	19.3 ± 0.07
TAN	mg/L	0.3 ± 0.06	0.3 ± 0.08	0.3 ± 0.08	0.2 ± 0.07	0.4 ± 0.05	0.5 ± 0.03
NO_2-N	mg/L	0.1 ± 0.02	0.1 ± 0	0.1 ± 0.01	0.1 ± 0.05	0.2 ± 0.02	0.2 ± 0.02
NO_3-N	mg/L	134 ± 3.7	133 ± 0.9	133 ± 5.6	59 ± 0	159 ± 1.3	280 ± 11
COD_{TOT}	mg O_2/L	50.4 ± 6.8	55 ± 15.1	77.5 ± 1.9	34 ± 6.3	65.3 ± 18.7	82.2 ± 7.8
COD_{DISS}	mg O_2/L	36.9 ± 2.4	38.2 ± 4.3	45.9 ± 6.8	15.7 ± 1.2	32.5 ± 1.5	44.2 ± 5.4
COD_{PART}	mg O_2/L	13.5 ± 9.24	16.8 ± 10.9	31.5 ± 4.9	18.2 ± 5.1	32.8 ± 17.2	38 ± 2.4
$BOD_{5\text{-}TOT}$	mg O_2/L	6.3 ± 1.1	5.3 ± 0.4	6.3 ± 2.2	2.1 ± 1.4	6.3 ± 2.8	7.19 ± 1.6
$BOD_{5\text{-}DISS}$	mg O_2/L	2.9 ± 0.2	3.2 ± 0.8	3.7 ± 0.04	0.4 ± 0.1	3.4 ± 0.8	5.8 ± 1.9
$BOD_{5\text{-}PART}$	mg O_2/L	3.4 ± 1.3	2.1 ± 0.3	2.5 ± 2.3	1.7 ± 1.2	2.9 ± 3.6	1.4 ± 0.3