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Deliverable D4.6:

Discharge from RAS

Test the relationship between feed composition and resulting discharge from RAS. Discharge will be characterized by concentrations and masses of relevant nutrients (N, P, O) and their different forms and fractions.

Authors:	Xiaoyu Huang, Sanni L. Aalto, Johanne Dalsgaard, Per B. Pedersen
WP/WP-leader:	WP 4.2/ Wout Abbink, Wageningen University and Research (WR), NL
Task/Task leader:	Task 4.4/Per Bovbjerg Pedersen, DTU Aqua, DK

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Executive summary

Trial 1 refers to the organic diets tested while trial 2 refers to the conventional diets.

Trial 1: Organic rainbow trout aquaculture uses fish trimming meals as a primary protein source. By using fish trimmings without bones/carcasses, dietary phosphorus can be reduced and by that P discharge. However, the effects of dietary phosphorus on phosphate concentration and microbiology in RAS are still unclear. This study, using four diets of different P content was performed in triplicated pilot scale RAS using rainbow trout at a feed loading of 1.67 kg feed/m^3 make-up water. The trial shows that resulting phosphate-P concentration in RAS was positively correlated to dietary phosphorus in RAS. Even though not statistically significant at the applied P-levels, the abundance of free-living microbes and the total microbial activity tended to decrease with low dietary P. However, TAN, nitrite-N, water clarity, microparticles, and organic matter were not affected by the diets. Results indicate that manipulating dietary P levels can be an effective way to reduce P discharge and potentially control microbes in RAS.

Trial 2: Soybean and rapeseed meals are popular alternatives for replacement of fishmeal in aquaculture feeds. Fermenting soybean and rapeseed meals are expected to improve feed digestibility. However, the effects of feed ingredients on water quality in recirculating aquaculture systems (RAS) are largely unknown, not to mention the effects of aforementioned plant-based meals. This study tested soybean meal (SC), fermented soybean meal (ST), rapeseed meal (RC), and fermented rapeseed meal (RT) in triplicated pilot scale RAS using rainbow trout. Results showed that the RT diet was characterized by high particulate organic matter and low water clarity, probably due to its relatively low digestibility and high FCR. Moreover, the RT diet also led to increased volume of microparticles, indicating poor faecal cohesiveness. The different diets did not affect microbial abundance, but lower microbial activity was found with the SC diet. This was probably due to the fact that a larger proportion of the organic matter derived was not biodegradable. Overall, results demonstrated that feed ingredients can affect both physicochemical and microbial water quality in RAS, and that fermenting rapeseed meal is not beneficial for RAS water quality.

Introduction

Trial 1: EU rules on organic aquaculture restrains the use of plant-based meals for organic rainbow trout (*Oncorhynchus mykiss*) production [1]. Thereby, fishmeal made from traditional trimmings is currently the primary protein source in nutrient-dense organic trout feeds even though with the drawback of containing excessive of phosphorus (P) contents. A new method of concentrating fish trimming meal to reduce dietary phosphorus by removing bone contents is currently being investigated.

For rainbow trout, the requirement, depending on FCR of available dietary phosphorus for growth and bone deposition is approximately 0.4–0.5% [2],**[3],**[4], generally corresponding to a dietary P-content of 0.6-0.7 %. However, different factors determine the level of available P in commercial feeds, including the limitations set by P-digestibility and body retention rate [5], as well as ingredient P-content, costs, and availability [6]. Currently, dietary P in commercial rainbow trout feeds vary between 0.7 and 1.2%, of which the excesses will be released into aquaculture systems as particulate P and/or dissolved phosphate. Phosphate excretion increases rapidly once the minimal requirement for available dietary P is met [2]- [3].

There is no internal treatment device for P removal in RAS and thereby phosphate can accumulate up to 20 mg P/L in RAS water [7], [8]. However, as an essential macronutrient, and in inorganic form readily available for microorganisms to utilize, phosphate can potentially influence microbial growth [9] and community structure [10]. Increased microbial abundance has already been demonstrated to cause a significant reduction in dissolved phosphate in RAS [11]. However, the effects of dietary P on phosphate concentration and microbial management in RAS is still unclear and this study was performed to elucidate this.

Trial 2: In recirculating aquaculture systems (RAS), nutrient waste is solely derived from the feed input. The dissolved and suspended waste and unremoved solid waste from fish can accumulate in RAS until an equilibrium level is reached [12]. The waste itself contributes to the physicochemical water quality and in addition it provides nutrients for living microbes. Thus, RAS water quality is expected to be influenced by feed composition and the ingredients used. This potential relationship is, however, still largely unstudied, and unknown.

A wide range of alternative protein sources and ingredients are proposed or applied in modern salmonid diets to substitute fishmeal. Soybean and rapeseed meals are commonly used plant-based protein compounds over the last five years [13]. Compared to fishmeal-based diets, the inclusion of plant-based meal can compromise feed digestibility due to nature of the ingredient and potential presence of anti-nutritional factors, which then leads to an increase in fecal waste produced by the fish [14]. Furthermore, fecal waste derived from plant-based meal tends to disintegrate more easily into fine solids due to reduced cohesiveness and sinking speed [15],[16],[17]. In RAS, such fine solids will accumulate since mechanical filtration is generally only targeting particles above some 50 µm. Previous studies have found higher concentrations of fine solids and organic matter with plant-based diets compared to fishmeal-based diets [17], [18]. However, studies comparing different plant-based meals is very limited. Nowadays, fermentation is often applied in feed producing process to improve feed digestibility. Several studies have tested the application of fermented ingredients focusing mainly on fish performance, while their derivative effects on RAS water quality till now remain unknown.

This study therefore aimed at examining the relationship between feed composition (protein source) and water quality parameters and associated microbiology in RAS. In addition, the effects of using fermented plant-based meals on RAS water quality was examined. Four different diets, developed and produced by Aller Aqua A/S as part of FutureEUAqua´s WP2, and containing 15% of either soybean meal, fermented soybean meal, rapeseed meal, or fermented rapeseed meal were tested in triplicated pilot scale RAS with rainbow trout (*Oncorhynchus mykiss*).

Materials and Methods

Feed composition

Trial 1: Four types of feeds were formulated to provide 46% of crude protein and 25% of crude fat (Table 1**Error! Not a valid bookmark self-reference.**). The high phosphorus (HiP) feeds contained 55% of fishmeal from fish trimmings, whereas the medium phosphorus (MeP) and low phosphorus (LoP) feeds replaced fishmeal with concentrated trimming meal (by further removing carcass remnants) at 10% and 20% of dietary protein (

Table *2*), resulting in different dietary P contents, i.e. 1.50%, 1.20%, and 0.90% respectively (Table 1). There was another control diet (HIP brewers' yeast free diet, HIPYF) containing high phosphorus (1.52%) but eliminated the content of brewers' yeast.

Table 1. Nutrient composition and digestibility of three types of experimental feeds: high phosphorus (HiP), medium phosphorus (MeP), and low phosphorus (LoP).

%	HiP	MeP	LoP	HiPYF
Nutrient composition				
Dry Matter	95.1	94.6	94.8	94.3
Crude protein	46.5	46.1	46.4	45.4
Crude fat	26.4	25.6	24.8	26.2
NFE[a]	12.5	14.9	17.4	12.8
Crude ash	9.69	7.94	6.20	9.8
Phosphorus	1.50	1.20	0.90	1.52
Nutrient digestibility determined in this trial				
Dry matter	76.6	78.9	79.6	81.6
Protein	86.6	88.2	89.3	89.7
Fat	79.7	82.5	79.3	84.5
NFE	55.5	60.9	65.1	72.3
Phosphorus	57.9	61.5	68.0	56.6

[a] Nitrogen-free extracts

Table 2. Ingredient compositions of four experimental diets.

Raw material (%)	HiP	HiPYF	MeP	LoP
Fish trimming meal	55.0	59.0	40.6	26.5
Concentrated fish trimming meal	-	-	10.6	21.2
Soybean cake	9.3	10.0	10.0	10.0
Fish oil from trimmings	17.3	17.3	17.9	18.5
Organic vegetable oil				
Wheat	10.0	13.4	12.6	15.5
Brewers' yeast	8.0	-	8.0	8.0
Vit/Min mix	0.35	0.35	0.35	0.35

Trial 2: Four different types of plant-based protein sources were examined in this study (Table 3). The RC diet contained rapeseed meal, the RT diet contained fermented rapeseed meal, the SC diet contained soybean meal, and the ST diet contained fermented soybean meal. The remaining ingredients were the same in all diets with only minor adjustments to balance the nutrient compositions to achieve iso-nitrogenous, iso-lipidic, and iso-carbohydrate feeds (Table 4).

Table 3. Ingredient composition of four experimental feeds: rapeseed meal (RC), fermented rapeseed meal (RT), soybean meal (SC) and fermented soybean meal (ST).

RC	Rapeseed	Fermented rape seed	Soybean meal	Fermented soybean meal
%	RC	RT	SC	ST
Rapeseed meal	15.0	-	-	-
Rapeseed meal fermented	-	15.0	-	-
Soybean meal	-	-	15.0	-
Soybean meal fermented	-	-	-	15.0
Fishmeal LT	22.5	22.5	20.0	20.0
Poultry meal	6.0	6.0	6.0	6.0
Feather meal	8.0	8.0	8.0	8.0
Haemoglobin	7.9	8.4	7.5	7.7
Rapeseed oil	12.1	12.3	12.8	12.8
Fish oil	8.0	8.0	8.0	8.0
Wheat	18.2	18.0	20.3	20.1
Diamol	1.0	1.0	1.0	1.0
Monoammoniumphosphate	0.49	0.00	0.50	0.51
L-Lysine	0.23	0.16	0.22	0.27
DL-Methionine	0.23	0.23	0.29	0.29
Vitamin premix	0.25	0.25	0.25	0.25
Mineral premix	0.15	0.15	0.15	0.15

Table 4. Analysed composition and digestibility of the four experimental feeds used.

%	RC	RT	SC	ST
Nutrient composition				
Dry matter	93.6	93.6	93.6	93.5
Crude protein	44.4	44.3	44.6	45.0
Crude fat	21.6	21.6	21.2	21.5
NFE	20.5	20.4	20.9	20.0
Crude ash	7.1	7.3	6.8	6.9
Phosphorus	1.00	0.85	0.91	0.88
Nutrient digestibility				
Dry matter	81.7	81.0	85.2	85.1
Protein	91.6	90.9	93.4	92.7
Fat	78.9	77.1	78.7	80.3
NFE	74.1	74.1	83.6	82.7
Phosphorus	67.8	73.3	72.6	75.9

Experimental setup and system operation

Trial 1: The trial consisted of 3 weeks of acclimation and 5 weeks of experiment. Twelve identical pilot RAS, as described in de Jesus Gregersen et al. (2021), were randomly assigned to 4 dietary treatments (in triplicate) at DTU Aqua, Hirtshals, Denmark. Each tank was stocked with 7.84 ± 0.02 kg of juvenile rainbow trout in freshwater with 100 g of feed per day. Water exchange rate was kept at 60 L per day, resulting in a feed loading of 1.67 kg feed/m^3 makeup water. Dissolved oxygen level was maintained between 85% to 100% by an automatic monitoring and dosing system (OxyGuard Pacific System, OxyGuard International A/S, Denmark). By adding sodium bicarbonate, pH was maintained between 7 and 7.4. Water temperature was kept at 16-17 °C and daily light/dark cycle was kept at 14h/10h. Feces settled in the sludge collectors were removed every day. No feed waste was observed in the sludge.

Trial 2: The trial consisted of 3 weeks of acclimation and 6 weeks of experiment. Twelve identical pilot scale RAS, described in de Jesus Gregersen et al. (2021), were randomly assigned to 4 dietary treatments (in triplicate) at DTU Aqua, Hirtshals, Denmark. The cylindroconical tank (500 l) in each RAS was stocked with 12.19 ± 0.16 kg of juvenile rainbow trout (Oncorhynchus mykiss) in freshwater and given 100 g of feed per day. Water exchange rate (make-up water) in each system was fixed at 60 L per day, resulting in a feed loading of 1.67 kg feed/m3 makeup water for all twelve RAS. Dissolved oxygen level was maintained between 85% to 100% by an automatic monitoring and dosing system (OxyGuard Pacific System, OxyGuard International A/S, Denmark). By adding sodium bicarbonate according to need, pH was maintained between 7 and 7.4. Water temperature was kept at 16-17 °C and daily light/dark cycle was kept at 14h/10h. Feces settled in the sludge collectors at the bottom of the cylindroconical tanks were removed every day. No feed waste was observed in the sludge.

Sampling and sample analysis

Trial 1: Grab samples were collected from the pump sump in each system prior to daily system maintenance and stored at 4°C until analysis. Fecal samples were collected over consecutive 3 days in week 6 and stored at -20°C until analysis. Sample analysis methods is shown in Table 5.

Table 5. Summary of water, feed, and fecal sample analysis. Microbial activity was described by H_2O_2 degradation rate (k value) and Bactiquant® (BQV value). Abbreviations: TAN = total ammonia nitrogen, BOD_5 = 5-day biochemical oxygen demand, COD = chemical oxygen demand.

Parameter	Filtration	Unit	Methods
TAN	0.20 µm	µg N/L	[19]
Nitrite-N	0.20 µm	µg N/L	[20]
Nitrate-N	0.20 µm	µg N/L	[21]
Total BOD5	unfiltered	mg O_2/L	[22]
Dissolved BOD5	0.45 µm	mg O_2/L	[22]
Total COD	unfiltered	mg O_2/L	[23]
Dissolved COD	0.45 µm	mg O_2/L	[23]
Microbial catalase activity	unfiltered	h^{-1}	[24]
Microparticles	unfiltered	#/mL	AccuSizer 780 SIS & Multisizer 4e Coulter Counter[1]
Turbidity	unfiltered	NTU	Hach 2100Q detector[2]
UV transmittance	unfiltered	%	UV spectrophotometer[3]
Free-living microbial abundance	40 µm	#/mL	[25]

[1]AccuSizer 780 SIS, Particle Sizing Systems, USA; Multisizer 4e Coulter Counter, Beckman Coulter Inc, USA.
[2]Hach 2100Q detector, Hach Lange, USA
[3]DU 530 Life Science UV/Vis, Bechman Coulter Inc., USA

Trial 2: Grab samples were collected from the pump sump of each system prior to daily system maintenance, filtered as needed and stored at 4°C until analysis. Fecal samples were collected over consecutive 3 days in week 6 and stored at -20°C until analysis. Analytic methods applied are shown in Table 6.

Table 6. Summary of water, feed, and fecal sample analyses. Microbial activity was expressed by H_2O_2 degradation rate (k value), as well as by Bactiquant® value (BQV). Abbreviations: TAN = total ammonia nitrogen, BOD_5 = 5-day biochemical oxygen demand, COD = chemical oxygen demand, KN = Kjeldahl nitrogen, TP = total phosphorus.

Parameter	Filtration	Unit	Methods
Water			
TAN	0.20 µm	µg N/L	[19]
Nitrite-N	0.20 µm	µg N/L	[20]
Nitrate-N	0.20 µm	µg N/L	[21]
Total BOD_5	unfiltered	mg O2/L	[22]
Dissolved BOD_5	0.45 µm	mg O2/L	[22]
Total COD	unfiltered	mg O2/L	[23]
Dissolved COD	0.45 µm	mg O2/L	[23]
Microbial activity	unfiltered	1/h	[24]
Microbial activity	unfiltered	BQV	[25]
Microparticles	unfiltered	-	AccuSizer 780 SIS & Multisizer 4e Coulter Counter[1]
Turbidity	unfiltered	NTU	Hach 2100Q detector[2]
UV transmittance	unfiltered	%	UV spectrophotometer[3]
Free-living microbial abundance	40 µm	#/mL	[25]
Faeces			
KN	unfiltered	%	[26]
TP	unfiltered	%	[27]
Fat	unfiltered	%	[28]
Dry matter & ash	unfiltered	%	[29]
COD	unfiltered	mg O_2/L	[23]

[1]AccuSizer 780 SIS, Particle Sizing Systems, USA; Multisizer 4e Coulter Counter, Beckman Coulter Inc, USA.
[2]Hach 2100Q detector, Hach Lange, USA
[3]DU 530 Life Science UV/Vis, Bechman Coulter Inc., USA

Statistics

Trial 1: Statistical analysis was performed in R. Based on the nitrate development, water quality data (except phosphate-P) from the last 2 week were chosen to enter statistical analysis in order to compare treatment effects. The differences between treatments were examined with one-way ANOVA followed by Turkey test for pairwise multiple comparisons. If the normality (by Shapiro-Wilk test) and variance homogeneity (by Bartlett test) were not met, Kruskal–Wallis test would be performed followed by Dunn test. Weekly comparison of water quality was examined by T test. Wilcoxon Signed-Rank test would be performed if the normality was violated. To test the effects of diets on phosphate along the time, two-way repeated measures ANOVA was used. The dietary effects at each time point were further tested by one-way ANOVA, followed by pairwise T test for multiple comparisons. Significant level was set at 0.05 and results were presented as mean ± standard deviation.

Trial 2: At week 5-6 the ST diet was all used and the SC diet was therefore applied as replacement for the remaining experimental days. Weekly data were standardized by subtracting corresponding values at the start of the experiment (week 0). The differences entered statistical analysis performed in R

(version 4.2.0). The effects of time and diet were tested by 2-way ANOVA, followed by 1-way ANOVA to explore the simple main effects of diets and pairwise T-test to conduct pairwise comparisons, both at weekly basis. The effects on FCR and fecal composition were tested through 1-way ANOVA, followed by Tukey test for pairwise multiple comparisons. For data that did not meet normality (by Shapiro-Wilk test) and variance homogeneity assumptions (by Bartlett test), Kruskal–Wallis test were applied followed by Dunn test. Results are presented as mean ± standard deviation throughout, and the level of significance was set at 0.05.

Results Trial 1

Phosphate

Dietary P significantly affected phosphate concentrations in the water of the 12 RAS throughout the experiment in a positive relationship (two-way ANOVA, $F_{3,48}$=505.0, p<0.001) (Figure 1). As it appears from the figure, the HiP, MeP, and HiPYF groups had sharp increases in the first 2 weeks and then approached a system equilibrium, whereas phosphate concentrations in the LoP group continued to decrease after 5 weeks of experiment. Between the HiP, MeP, and LoP groups, phosphate-P concentrations increased with dietary P.

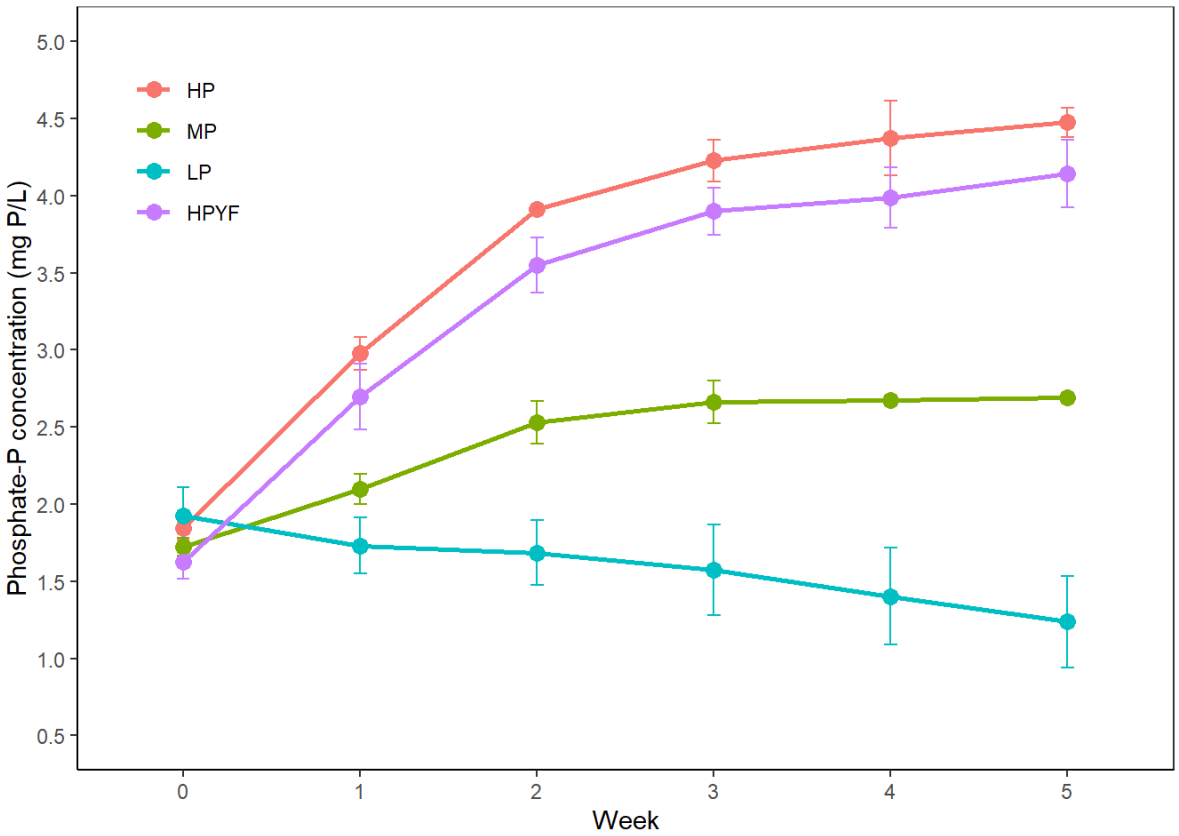

Figure 1. The development in phosphate-P concentrations in RAS water for four diets over 5 weeks.

FCR and chemical water quality

Feed conversion rates (FCR) were not affected by the diets (one-way ANOVA, $F_{2,6}$=3.2, p>0.05), ranging between 0.9 and 1.0 (Table 7). TAN and nitrite did not differ between treatments in the final 2 weeks (TAN: one-way ANOVA, $F_{3,20}$=2.5, p>0.05; nitrite: $F_{3,20}$=0.6, p>0.05), being approximately 120 and 85 µg N/L (Table 7). Nitrate, on other hand, was a little affected (Kruskal-Wallis, H_3=12.6, p=0.006). The highest nitrate concentration was found with the LoP diet, but only 4% higher than the lowest in the HiPYF group. All systems had very low water clarity (Table 7). In the final 2 weeks, turbidity was not affected (one-way ANOVA, $F_{3,20}$=2.6, p>0.05) by diet while UVT was significantly higher with the LoP diet ($F_{3,20}$=11.0, p<0.001). The highest UVT was found in the HiPYF group while the lowest was found in the HiP group. Furthermore, the diets did not affect microparticle abundance and β value.

Regarding organic matter, BOD_5 was not affected (particulate BOD_5: one-way ANOVA, $F_{3,20}$=2.0, p>0.05; dissolved BOD_5: Kruskal-Wallis, H_3=6.1, p>0.05) and COD (particulate COD: one-way ANOVA, $F_{3,20}$=2.1, p>0.05; dissolved COD: $F_{3,20}$=12.0, p<0.001) was little affected by the diets (Table 8). Only the HiP group had higher dissolved COD than the other groups in the final 2 weeks, while the differences were less than 3.5 mg O_2/L.

Table 7. Summary of FCRs (n=3) and average water quality from the final 2 weeks (n=6) (mean ± standard deviation) under the different diets. Lowercase letters indicate statistical differences from other treatment groups.

Parameter	HiP	MeP	LoP	HiPYF
FCR	0.98 ± 0.03	0.93 ± 0.02	0.94 ± 0.02	0.92 ± 0.02
TAN (µg N/L)	119 ± 21	115 ± 10	138 ± 16	135 ± 20
Nitrite-N (µg N/L)	81 ± 11	85 ± 13	87 ± 16	78 ± 11
Nitrate-N (mg N/L)	60.6 ± 1.2^{ac}	59.6 ± 1.1^{bc}	61.4 ± 1.1^{a}	59.0 ± 0.7^{b}
Turbidity (NTU)	7.3 ± 1.5	7.9 ± 1.5	7.7 ± 1.2	5.7 ± 1.7
UVT (%)	47.2 ± 2.2^{c}	48.3 ± 2.8^{bc}	51.5 ± 1.5^{ab}	53.6 ± 2.1^{a}
Free-living microbes (million/mL)	38.2 ± 26.2	36.2 ± 27.1	18.5 ± 3.4	18.4 ± 5.9
Microbial catalase activity (h^{-1})	0.62 ± 0.42	0.67 ± 0.36	0.40 ± 0.05	0.29 ± 0.07
Microparticle β value	3.42 ± 0.24	3.43 ± 0.16	3.33 ± 0.16	3.46 ± 0.08
Microparticle (million/mL)	1.03 ± 0.82^{a}	1.13 ± 0.79^{a}	0.53 ± 0.15^{ab}	0.38 ± 0.10^{b}

Table 8. Summary of BOD_5 and COD from the final two weeks (n=6) (mean ± standard deviation). Lowercase letters indicate statistical differences from other treatment groups. Unit: mg O_2/L

Parameter	HiP	MeP	LoP	HiPYF
Particulate COD	18.1 ± 4.8	18.2 ± 3.1	20.4 ± 4.5	13.4 ± 5.3
Dissolved COD	29.5 ± 1.0^{a}	27.5 ± 0.7^{b}	26.9 ± 1.0^{b}	26.3 ± 1.4^{b}
Particulate BOD_5	3.9 ± 1.0	4.5 ± 0.8	4.6 ± 0.9	3.4 ± 1.0
Dissolved BOD_5	1.6 ± 0.2	1.4 ± 0.3	1.3 ± 0.2	1.4 ± 0.2

Microbial water quality

The abundance of free-living microbes varied greatly between the groups and with time (Figure *2*a). In the last 2 weeks, the average abundances in the HiP and MeP group (38.2±26.2 and 36.2±27.1 million/mL, respectively) were double that of the LoP and HiPYF group (18.5±3.4 and 18.4 ± 5.9 million/mL) (Table *7*7). However, the differences were not statistically significant.

Microbial activity in terms of H_2O_2 degradation k values exhibited high weekly variations in all treatment groups (Figure *2*b), similar to the microparticle data. In the final 2 weeks, the average H_2O_2 degradation k value with the LoP diet (0.40±0.05/h) was 30% to 40% lower than those with the MeP and HiP diets (Table 7). However, the differences were not significant.

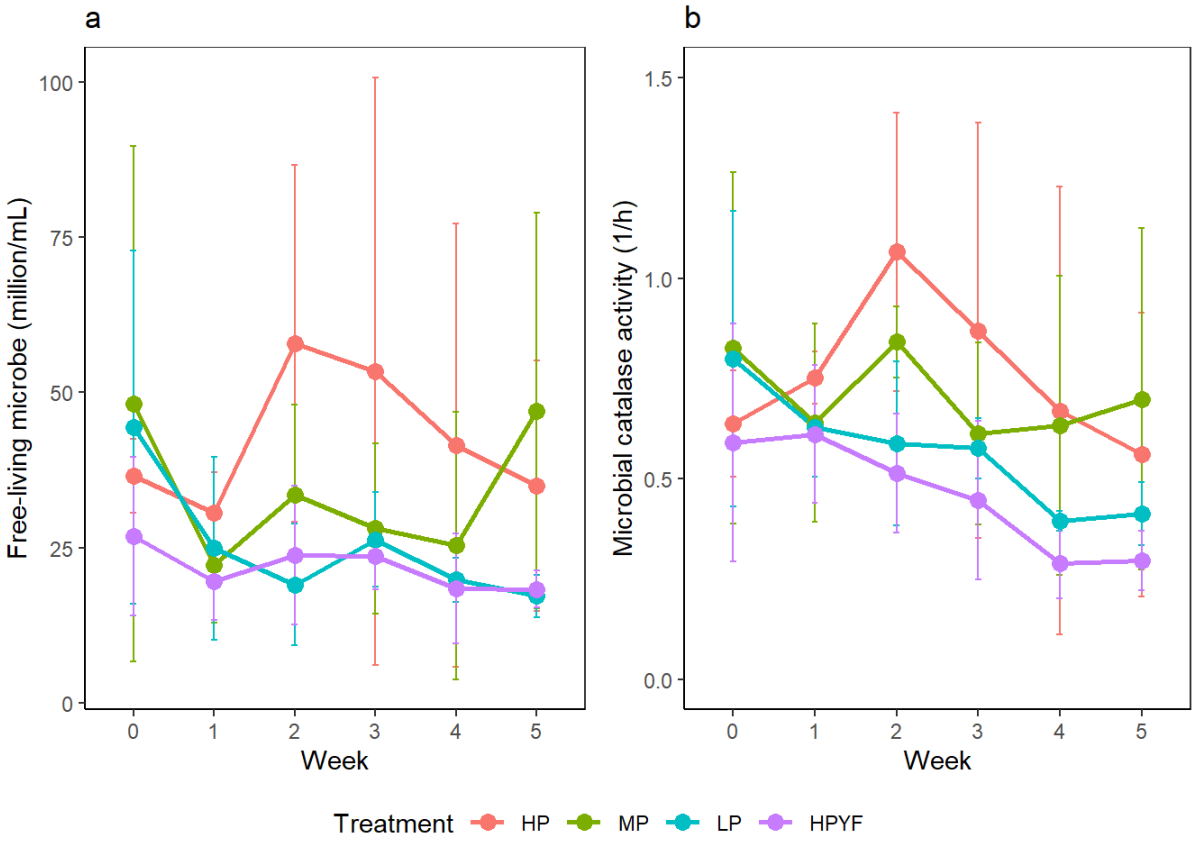

Figure 2 a & b. The weekly development of (a) abundance of free-living microbes and (b) microbial activity (H_2O_2 degradation k value) in RAS (mean ± standard deviation) (n=3).

Discussion Trial 1

Reducing dietary P concentrations can be an effective approach to reduce overall P waste in aquaculture given that fish P requirements are met [6], [30]. The current study demonstrated that this also applies to phosphate-P accumulation in intensive RAS. Reducing total dietary P content from 1.49 to 0.89% thus reduced system phosphate-P concentrations by the end of the trial by approximately 70%, i.e., from 4.43 to 1.32 mg PO_4-P/L at a feed loading of 1.67 kg feed/m3 make-up water. In the current study, fish P requirements were met by all diets and FCRs were similar and below 1 in all treatment groups.

Standardized, available dietary P concentrations (calculated by multiplying dietary P concentration by FCR and ADC of P from the mass-balance study) were all above or at the anticipated minimum requirement concentration, decreasing from 8.9 g/kg dry feed in diet HiP to 6.0 g/kg dry feed in the Lo3P diet. Previous studies have shown that it is possible to obtain near-zero phosphate-P excretion from rainbow trout without compromising fish performance by approaching available dietary P concentrations to minimum requirement levels [4], [31]. Consequently, the concentration of phosphate-P in RAS water, and thereby the phosphate discharge from RAS can be quite accurately controlled by the composition of the feed applied.

Independently of diet, the measured phosphate-P concentrations in RAS water were approximately 60% lower than the theoretical equilibrium concentrations reflecting the phosphate-P excreted directly by the fish. The lower phosphate-P concentrations observed were most likely associated with microbial utilization including P-uptake by microorganism in biofilms and in water of the RAS. As an essential macronutrient, phosphate could potentially alter microbial abundance, composition, and activity [9], [10] in RAS.

In this case a feed loading of 1.67 kg feed/m^3 make-up water, corresponding to 667 L MUW/kg feed applied, was used. As described above, reducing total dietary P content from 1.49 to 0.89% reduced the resulting phosphate-P concentrations in RAS water from 4.43 to 1.32 mg phosphate-P/L. This means, that while dissolved phosphate discharge from HiP-systems would be 2.95 g PO_4-P/kg feed applied (667 L/kg feed X 4.43 mg PO_4-P/L) it would only be 1.1 g PO_4-P/kg feed applied (667 L/kg feed X 1.67 mg PO_4-P/L) from systems using LoP feed. A reduction of 1.85 g PO_4-P/kg feed applied corresponding to a 63 % reduction in discharge of phosphate.

Nutritional strategies applied to reduce the discharge of phosphorus or to manage microbial status in RAS should not compromise the performance of nitrifying bacteria in the biofilters. In this study, no differences in TAN or nitrite concentrations were observed, demonstrating that the P-levels obtained in the systems did not harm the nitrification process.

Conclusion Trial 1

By testing diets with different levels of phosphorus, this study confirmed that resulting phosphate concentration in RAS water is positively correlated to dietary phosphorus. Applying low dietary P did not largely affect physicochemical water quality parameters or nitrification performance. This study demonstrates that lowering dietary P levels can be a feasible and effective way to reduce P discharge from RAS.

Results Trial 2

Inorganic nitrogen contents and phosphate

The diets affected TAN and nitrate concentrations (2-way ANOVA, $F_{3,44}$=10.38, p<0.001; $F_{3,44}$=39.26, p<0.001; $F_{3,44}$=29.89, p<0.001; Fig. 3). The significant difference in TAN concentrations was found at

week 5 (1-way ANOVA, $F_{3,6}$=22.15, p=0.002), where TAN was 3 times higher with the SC diet than that with the RC and RT diets (pairwise T test, p=0.001 and p=0.001, respectively). Nevertheless, all TAN concentrations were below 0.15 mg TAN/L. Nitrite concentrations was not affected by the diets (2-way ANOVA, F3,44=2.54, p=0.068), and the concentrations were all below 0.15 mg NO_2-N/L.

Nitrate accumulated continuously throughout the trial and reached 75 - 80 mg NO_3-N/L at week 6. The dietary effects could be detected as early as the first week (1-way ANOVA, $F_{3,8}$=7.58, p=0.010), where the RT diet (having the lowest amount of dietary protein) had the least nitrate accumulation. There was no statistical difference between the other 3 diets.

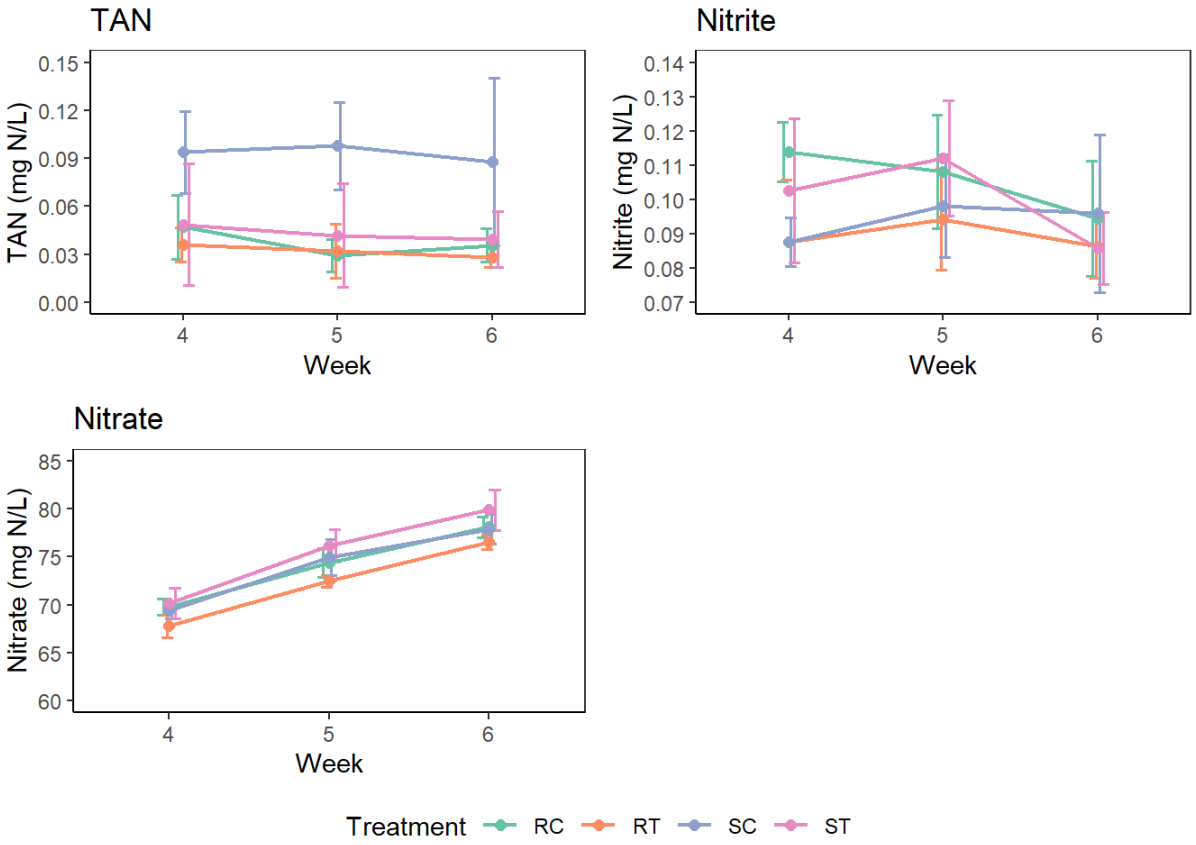

Figure 3. Weekly development in TAN, nitrite-N, and nitrate-N concentrations.

Water clarity

Turbidity was already high (around 15 NTU) and UVT was already low (around 32%) at the start of the trial (week 0), and they continued to worsen (2-way ANOVA, $F_{5,44}$=3.06, p=0.019; $F_{5,44}$=4.59, p=0.002) with turbidity increasing approximately 5 NTU and UVT decreasing approximately 2%. There was no dietary effect on turbidity (2-way ANOVA, $F_{3,44}$=0.95, p=0.423) but significant dietary effect on UVT ($F_{3,44}$=5.38, p=0.003) was found between the RT (26.51 ± 2.72%) and SC group (31.77 ± 1.80%) in week 5 (p=0.034).

Microbial abundance and activity

At weeks 4-6, the abundance of free-living (FL) microbes varied between 10 - 25 million/mL, and the total surface area of microparticles (potentially serving as substrate for particle-attached microbes) in the range 5 - 100 µm varied between 22 - 35 mm^2/mL. However, the abundance was unaffected by the diets throughout the whole trial (2-way ANOVA, $F_{3,44}$=2.01, p=0.127; $F_{3,44}$=2.30, p=0.090). Microbial activity (described by the H_2O_2 degradation rate constant k (h^{-1})), on the other hand, was affected (2-way ANOVA, $F_{3,44}$=3.82, p=0.016). In week 4, the SC group had the lowest activity (1.03 ± 0.10/h), being approximately 0.6 - 0.7/h lower than the RC, RT, and ST groups (pairwise T test, p=0.008, p=0.029, and p=0.027, respectively). In week 5, the microbial activity was still lower with the SC diet than that with the RT diet (p=0.012). Changing from ST to SC diet (week 5-6) did not cause a concomitant drop-in activity (Suppl. Figure 4). Microbial activity described by BactiQuant® showed no statistical difference between treatments (2-way ANOVA, $F_{3,44}$=1.15, p=0.339). However, there was a tendency for the SC group to have a lower activity here as well.

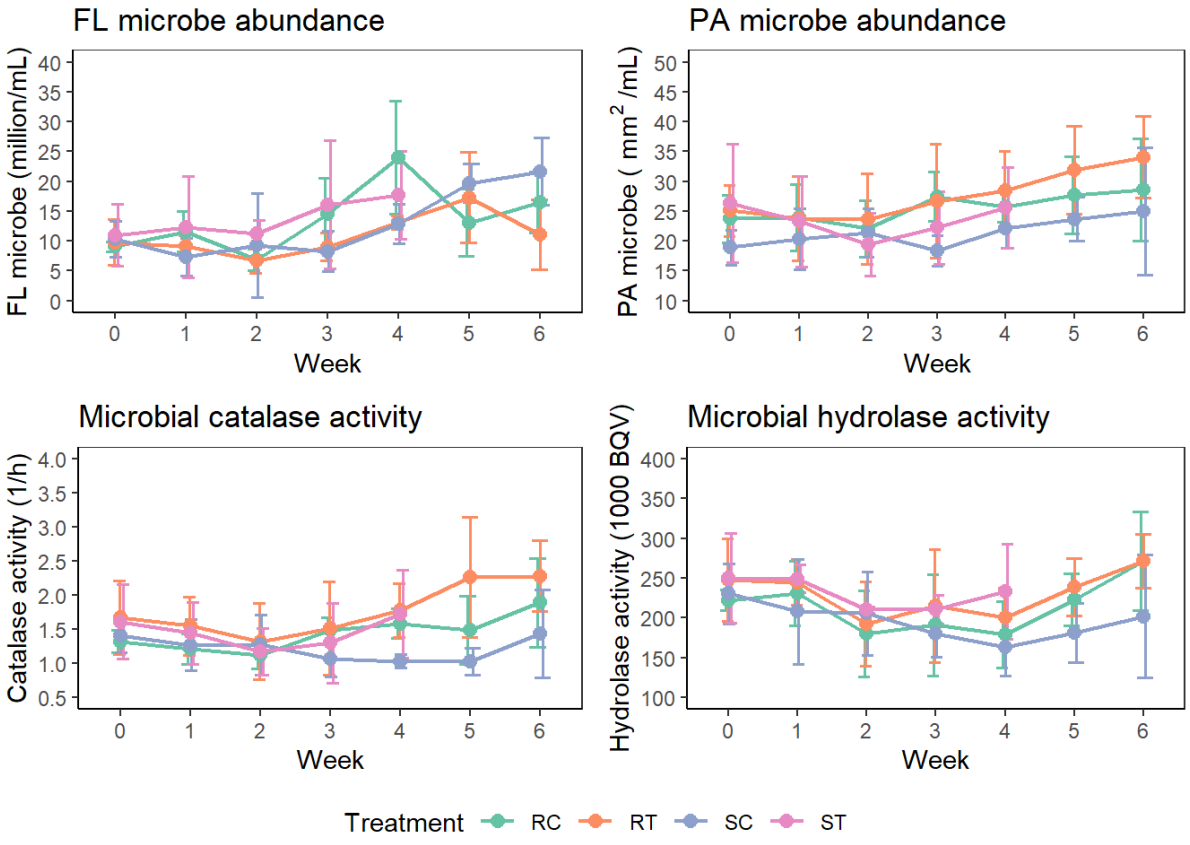

Figure 4. Weekly development in microbial abundance and activity.

Microparticles

Small microparticles (1 - 5 µm) did not statistically differ in abundance between treatments (2-way ANOVA, $F_{3,44}$=0.93, p=0.436). The abundance of larger microparticles (5 - 100 µm) was affected by the diets ($F_{3,44}$=3.12, p=0.036). However, the pairwise T-test could not detect group difference. Furthermore, the microparticle size distribution was not affected either since the beta value, being 2.7

– 2.9 did not change between treatments ($F_{3,44}$=1.04, p=0.385). Microparticle volume was a little affected in the larger size range ($F_{3,44}$=3.52, p=0.023), with the RT group (0.114 ± 0.036 mm^3/mL) having a higher particle volume than the SC group (0.063 ± 0.018 mm^3/mL) in week 5 (pairwise T test, p=0.032). There was no statistical difference in the volume of small microparticles (2-way ANOVA, $F_{3,44}$=0.76, p=0.522).

Organic matter

Total BOD_5 in RAS water was affected by the diets (2-way ANOVA, $F_{3,44}$=5.25, p=0.003) with the RT group (18.61 ± 4.49 mg O_2/L) having a higher total BOD_5 than the SC group (12.74 ± 1.87 mg O_2/L) at week 5 (pairwise T test, p=0.015) (Fig. 5). Within BOD_5, the particulate fraction was affected (2-way ANOVA, $F_{3,44}$=4.96, p=0.005), while the dissolved fraction remained unaffected by diet ($F_{3,44}$=0.71, p=0.554). The difference in particulate BOD_5 was also found between the RT (16.97 ± 4.74 mg O_2/L) and SC groups (10.89 ± 2.23 mg O_2/L) at week 5 (pairwise T test, p=0.016).

Total COD in RAS water was a little affected by the diets as well (2-way ANOVA, $F_{3,44}$=3.11, p=0.036). The difference was found between the RC (90.83 ± 11.16 mg O_2/L) and RT diets (101.70 ± 10.48 mg O_2/L) at week 5 (pairwise T test, p=0.029). Particulate COD was also affected (2-way ANOVA, $F_{3,44}$=4.02, p=0.013). The RT diet led to a higher particulate COD (59.70 ± 12.18 mg O_2/L) than the RC (49.50 ± 9.56 mg O_2/L) and SC diet (43.63 ± 5.89 mg O_2/L).

The diets also affected the level of biodegradability i.e., the ratio of total BOD_5/COD of the RAS water (2-way ANOVA, $F_{3,44}$=17.31, p<0.001). This ratio was found to be lower for the SC diet as compared to the RC and RT diets at weeks 3-5 (pairwise T test, p=0.006 & 0.025 at week 3, both p<0.006 at week 4, and p=0.029 & 0.016 at week 5). Moreover, the ST group had a lower ratio than the RC group at week 3 (p=0.016) and a higher ratio than the SC group at week 4 (p=0.004).

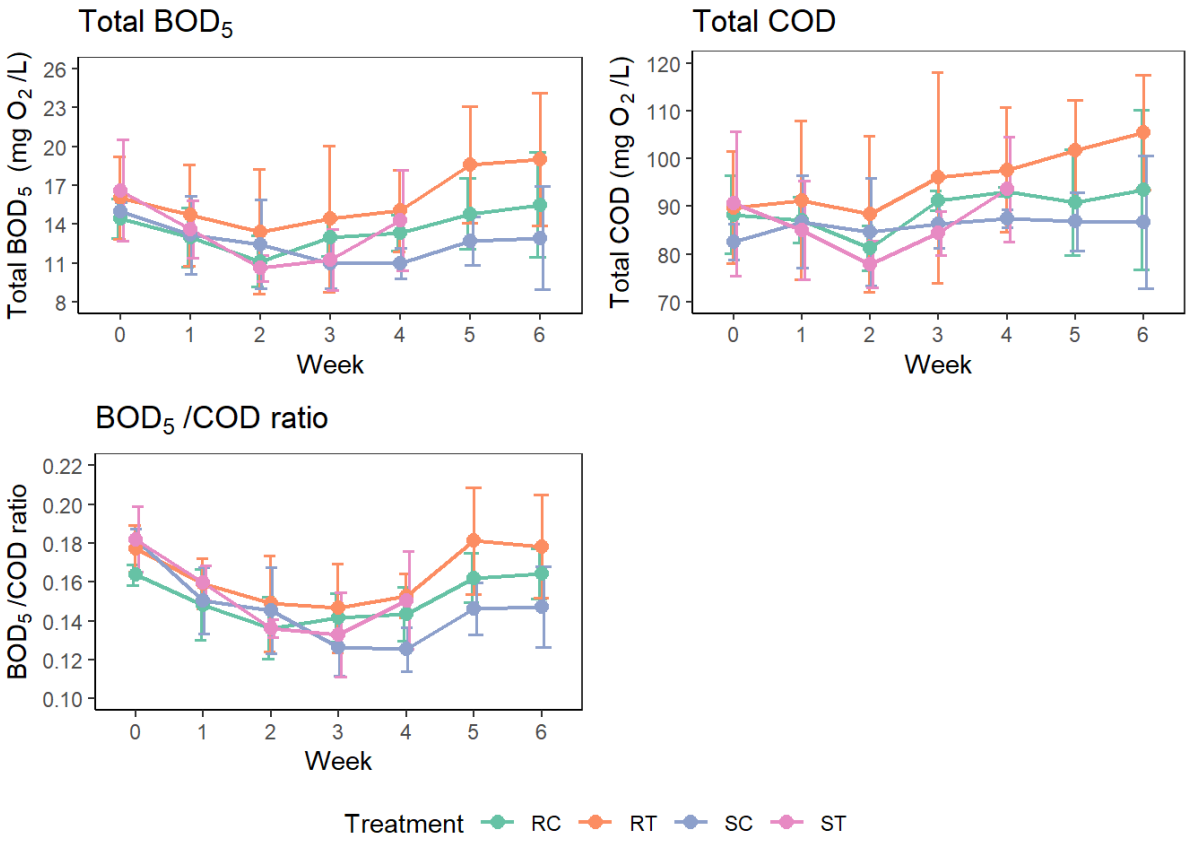

Figure 5. Weekly development of organic matter in RAS water.

FCR and fecal characteristics

There was no statistical difference in FCR between the RC, RT, and SC diets (1-way ANOVA, $F_{2,6}$=1.00, p=0.421), listed as 1.61 ± 0.08 (RC), 1.65 ± 0.02 (RT), and 1.55 ± 0.12 (SC). As previously described, the ST group experienced diet change (to SC) at weeks 5-6, leaving the FCR (1.47 ± 0.06) close to that of the SC group ($F_{1,4}$=1.12, p=0.35). No mortality was observed during the trial.

Fecal characteristics were affected by the diets (Table). There were no differences in fecal nutrient mass of dry matter (DM), Kjeldahl nitrogen (KN), and COD between the RC and RT groups, while being significantly higher than those of the SC group (1-way ANOVA, $F_{2,6}$=64.2, p<0.001; $F_{2,6}$=12.6, p=0.007; $F_{2,6}$=5.5, p=0.044, respectively). The RC group had the highest fecal TP content ($F_{2,6}$=35.2, p<0.001). Regardless of the differences of fecal KN mass, its proportions in fecal DM did not differ between dietary treatments ($F_{2,6}$=0.5, p=0.632).

Table 9. Summary of the characteristics of collected fecal matter for the RC, RT, and SC diets, respectively.

Diet	RC	RT	SC	unit

Nutrients collected in collectors at the bottom of the tanks				
DM	213.1 ± 11.0[a]	212.2 ± 5.3[a]	131.7 ± 12.5[b]	mg/g feed
KN	8.07 ± 0.77[a]	8.62 ± 0.59[a]	5.25 ± 1.18[b]	mg/g feed
TP	3.39 ± 0.12[a]	2.31 ± 0.07[b]	2.52 ± 0.25[b]	mg/g feed
COD	218.7 ± 72.1[ab]	282.7 ± 10.4[a]	164.5 ± 19.8[b]	mg O_2/g feed
Nutrient composition				
KN	3.79 ± 0.22	4.06 ± 0.20	3.96 ± 0.51	% of DM
TP	1.59 ± 0.03[a]	1.09 ± 0.01[b]	1.91 ± 0.03[c]	% of DM

Discussion Trial 2

In earthen fish ponds, rapeseed meal has been found to result in a better turbidity than soybean meal [32]. However, the difference was attributed to the precipitation of clay colloids caused by uneaten feed. In this experiment we found that the RAS fed RT diet were characterized by higher particulate organic matter and reduced water clarity. This was probably due to the relatively low digestibility of macronutrients (i.e., protein, lipid, and carbohydrate), which resulted in elevated fecal production and thereby more solid waste and particles accumulating in the rearing water. The higher (although not significant) FCR of the RT diet was probably also associated with the reduced digestibility of the diet. Different feed compositions also led to differences in fecal mass and compositions, in accordance with a study on European seabass (*Dicentrarchus labrax*) [33]. Here, we found that soybean meal caused less fecal matter collected than both rapeseed meals, again probably due to differences in digestibility Table 4 and [33].

Previously, plant-based ingredients have been found to cause higher abundance of microparticles (< 30 µm) than fishmeal [17], [34]. We observed no difference in microparticle abundance between the different plant-based diets although higher volumes of microparticles (5 – 100 µm) in the RT group could still indicate reduced cohesiveness of faeces when using fermented rapeseed meal.

In general, TAN and nitrate-N concentrations in RAS after biofilter nitrification are associated with total ammonia loading [1], [23]. The lower nitrate-N concentrations with the RT diet were due to reduced ammonia loading, induced by its lower protein digestibility. The SC diet led to the highest TAN concentrations in rearing water, in accordance with a previous study in earthen fish pond comparing soybean and rapeseed meals [32]. Anyway, the TAN concentration with the SC diet did not exceed 0.15 mg N/L.

Although organic matter concentrations were not statistically different between treatment groups still the highest values were observed for rapeseed with soybean meal (SC) being lowest in BOD_5 and COD

as well as biodegradability (BOD_5/COD ratio) (Fig. 5). This is well in line with the better digestibility of the soybean meal diets (Table 4) that thereby generates less particulate waste (COD and BOD_5) to the RAS water.

Organic matter is typically a major factor influencing microbial activity [11], [35]. The diets did not clearly affect the abundance of FL and PA microbes but the microbial activity, measured by H_2O_2 degradation rate as well as Bactiquant®, was found to be lower with the soybean (SC) diet (Fig. 4) which is in line with the above.

Conclusion Trial 2

This study investigated the relationship between four protein sources in fish feed and RAS water quality. The results indicate that feed composition can affect water quality in RAS. Using rapeseed resulted in increased organic matter content and reduced water clarity of RAS water whereas un-fermented soybean meal (SC) resulted in lower BOD_5 and COD as well as biodegradability (BOD_5/COD ratio). The associated microbial activity was also lower for the SC diet. Overall, results suggest feed composition to potentially be an important factor in diets to be used in recirculating aquaculture systems.

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