The Benefish Consortium reports on

The influence of system water refreshment rates on realized feed load, weight development, fish physiology and behaviour in turbot

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1 Introduction

Farmers with recirculation aquaculture systems (RAS) have a greater necessity and capacity to control the culture conditions of their farms than farmers with other aquaculture systems. Water quality is one of the factors that is closely monitored and managed in order to maintain the optimal levels of oxygen, ammonia, temperature, pH, and CO2. Effects of these parameters on growth and health are well studied and almost immediately noticeable. In RAS it often occurs that, although water quality conditions seem to be optimal, the feed intake of the fish might suddenly diminishes, thus reflecting a situation of sub optimal welfare of the animals. This phenomenon is particular relevant in marine RAS where these situations of reduced feed intake occur even though the normally monitored water quality parameters and husbandry conditions appear to be optimal. Similar phenomena also occur in other aquaculture culture systems, such as flow through systems, where feed intake fluctuates whilst the reasons are not always known, although there is typically less control and monitoring compared with RAS. It is therefore necessary to actively monitor deviation of expected feed intake, in combination with the monitoring of culture conditions and farm management on pilot-scale level. Only through this intermediate level experimental work and farm observations for the assumed relationship between deviation of expected feed intake and fish welfare can be validated. It is furthermore necessary to provide refinements to causative relationships expected to be found on commercial farms, where it is often claimed that e.g. lower system water refreshment rates or more closed RAS are leading to growth retardation and lower feed intake in fish and thus lower production. The present study is, therefore, intending to prove the hypothesis that changes in feed intake can be associated with changed fish welfare status, using turbot as model species. It is furthermore hypothesized that this changed fish welfare status is caused by different system water refreshment rates and fish and system management. As a final result, feed intake should relate by same efficiency to lower fish growth in closed RAS compared to flow through systems. The objectives are therefore to validate the relationships between deviation from expected feed intake and fish welfare, and their causative factors on the commercial farms interpreting data on feed intake, behavior, endocrinology and immune patterns as welfare indicators.

2 Materials and Methods

2.1 Husbandry, experimental systems and experimental period

Three pilot scale culture systems, consisting of six tanks each, were stocked with two different size classes of turbot. Two systems were RAS systems and one system was a flow through system. The dimensions of the systems are given in Table 1 and the related side view of the recirculation systems in Figure 1 and the topview on the facility in Figure 2 .

Table 1: system dimensions and characteristics of the three used cultured systems, 2 RAS and one flow through system

	RAS 1%	RAS 5%	Flow through
Total system volume (m³)	25.69	24.53	16.8
Tank volume (m³)	2.75	3.00	2.80
Tank surface area (m²)	5.3	5.3	5.3
Tank flow rate (m³/h)	2.27	3.00	2.24
Tank hydraulic retention time (tankvolume/h)	1	1	0.8
Averaged system refreshment rate (m³/kg feed)	1.4	5.0	71
Averaged system refreshment rate (% of total volume/h)	0.9	3.8	80
Volume biofilter (m³)	3.87	3.87	-
Drum filter mesh size (µm)	30	30	-
U.V. (W)	450	450	-
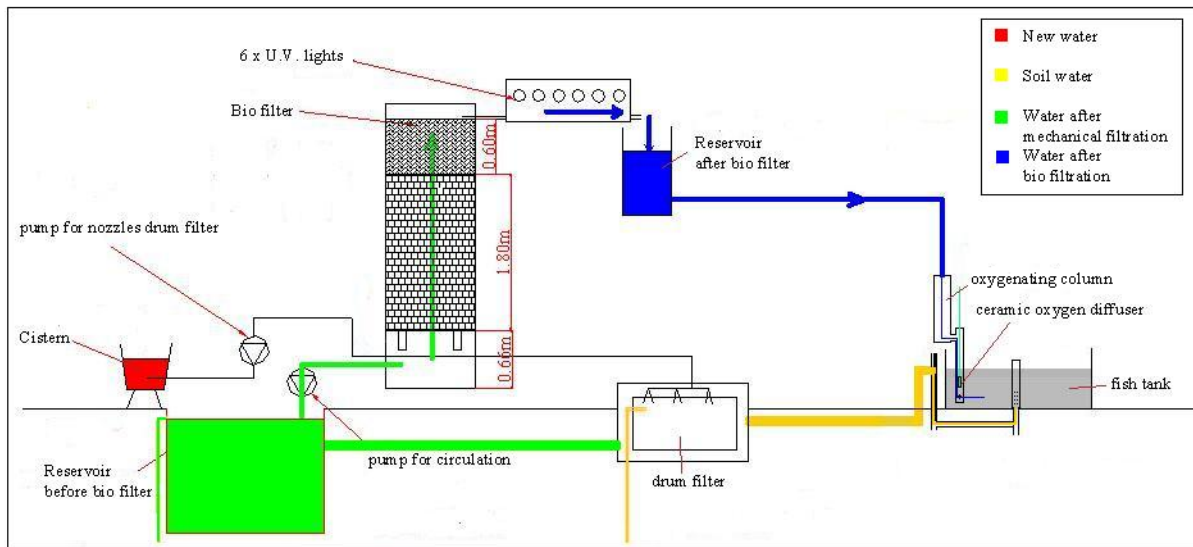
Figure 1: Schematic side view of the recirculation system layout
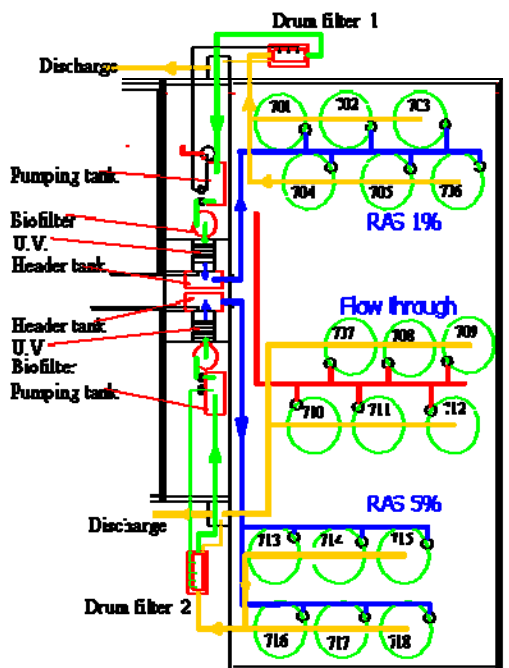
Figure 2: Schematic top view of the experimental facility with the three systems, two recirculation systems and one flow through system.

The difference between the two RAS systems was the amount of daily water refreshment. In first part of the experiment one system was refreshed with 1.2% of the total system volume per hour (averaged 1.4m³/kg feed) and the other with 3.8% (averaged 5.0m³/kg feed). The water refreshment rate in relation to the feed load differed as the percentage was kept stable during the experiment but fish biomass and therefore feed load

increased gradually. The exchange rate was calculated on monthly base. In the second part of the experiment, which was a direct continuation of the first part using the same systems and fish, the water refreshment rates was reduced in one RAS from 1.2% to 0.3% (Figure 3). This intended to increase the potential effect of low water freshmen rates on fish welfare. This management measure was taken on day 396 of the experimental period. These three RAS water refreshment rates are typical for commercial RAS systems that use rates between 500l to several m³/ kg feed.
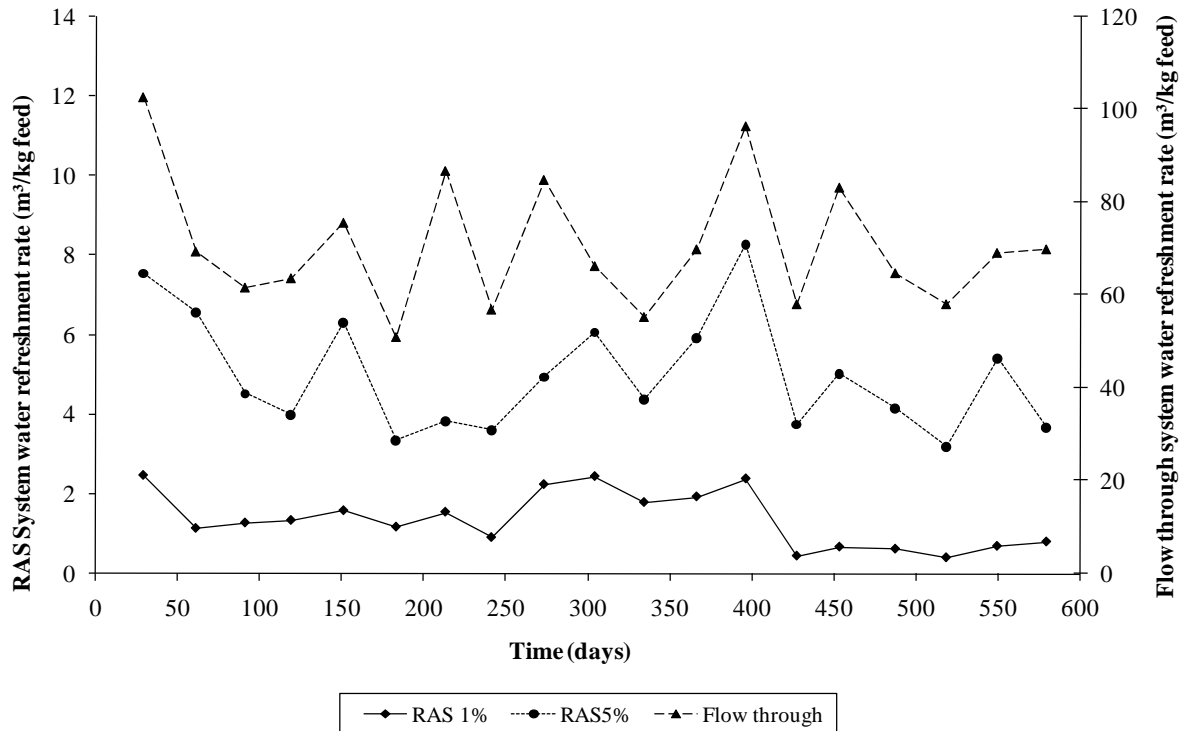
Figure 3: System water refreshment rates of the two recirculation aquaculture systems (RAS 1% and 5%) and the flow through system used in the study

The experimental period was from 1st of March 2007 till 1st of September 2008 and lasted for almost 600 days.

2.2   Experimental animals

At the beginning of the experimental period (1st March 2007) three tanks of each system were stocked with one size class of turbot (referred to as "large" or L) with an average weight of 518 ± 119 g and three tanks of each system with another age class (referred to as "small" or S) with an average weight of 183 ± 37 g. The averaged weights of inside these two size classes were not different (Two-way ANOVA, p=0.99). Within the two size classes the fish was divided into three subclasses, stocking the smaller (S) the medium (M) and heaviest (L) animals of the respective size class in different tanks, resulting in three subclasses per size class in total six subclasses. The resulting six subclasses are therefore: SS, SM, SL and LS, LM, LL. The detailed average weights per tanks are given in Table 2.

Table 2: Distribution of the fish size classes and their initial averaged individual stocking weight

System	Size class	subclass	Shortening	Tank	Average weight (g)
RAS 1%	Large	small	LS	701	375
		medium	LM	702	507
		large	LL	703	659
	Small	small	SS	704	143
		medium	SM	705	184
		large	SL	706	229
Flow through	Large	small	LS	707	384
		medium	LM	708	504
		large	LL	709	654
	Small	small	SS	710	146
		medium	SM	711	175
		large	SL	712	227
RAS 5%	Large	small	LS	713	401
		medium	LM	714	513
		large	LL	715	669
	Small	small	SS	716	138
		medium	SM	717	179
		large	SL	718	226

During the experimental period fish was removed from each tank in order to maintain a commercially applied fish density of 30 to 40 kg/m² in May 2007 and January 2008. The effect of this thinning is reported in the result section.

2.3 Feeding

Fish were hand feed three meals per day at 9.30h, 13.30h and 17.30h respectively. The individual tank feeding period lasted till saturation was observed; a typical duration was about 30minutes. The used feed was Le Gouessant, Turbot Label Rouge, France (Table 3).

Table 3: Composition of Turbot Label Rouge feed

Composition	Amount %
Fish meal	54.3
corn gluten	9.0
wheat gluten	6.4
Fish oil	5.7
water	10
Crude protein	55.0
Crude fat	12.0
Cellulose	0.56
ash	10.28
Phosphate	1.6

Feed load was recorded on monthly base by registering the amount of feed given per tank. Feed loss or spillage was not quantified but was limited to a minimum by the hand feeding.

2.4 Measurements and Samplings

2.4.1 Fish weight development

Fish weight development was recorded on monthly base by separating the individual tanks in two halves and gathering all fish at one side of the net. Then, 50 fish per tank were netted randomly and weight. Weighed fish was put on the other side of the net to avoid sampling the same individual twice. Dead fish was collected and recorded on daily base.

2.4.2 Water quality

Water quality was maintained within the range acceptable for fish and was measured for oxygen (Oxyguard Handy Gamma) and water temperature (Hach Lange HQ 40D) in each tank on daily base. pH (Hach Lange HQ 40D), total ammonia nitrogen and nitrite were measured both photometrical on system level three times per month.

2.4.3 Physiological parameters

During the experimental period fish were sampled for physiological parameters: condition factor, cortisol, blood plasma glucose and lactate concentrations, hematocrit and spleen weight. First sampling took place on 13th November 2007 (day 258) using 5 fish per tank and the second on 2nd 2008 (day 552) sampling 10 fish per tank. The number of sampled fish was increased due to the high data variation recorded during the first sampling. The second sampling happened therefore 4-5 months after the system water refreshment rate of RAS 1% was decreased (day 396). Fish were sacrificed by a blast to the head. Simultaneous sampling of the fish from one tank and the direct termination allowed minimizing bias of the obtained value due to netting stress. Blood samples were taken after fish were weight individually by a, with heparin coated, injection syringes and were tapped under the vertebra close to the tail region. Samples were stored on ice. After sampling blood cells and plasma were separated by centrifugation (8min by 10000rpm). Plasma samples were separated due to analytical requirements and store at -80C on dry ice and then transferred to a freezer at -70C till analysis. Hematocrit was determined in duplo per sample by filling capillars with blood and centrifuging them for 3min at 10000rpm. Differences in the heights of the columns of plasma and blood cells were measured with an accuracy of 0.1mm. Spleen weight was determined by removing the spleen from the fish by dissection and weighing the entire organ. Cortisol was measured by RIA (radio-immuno-assay, MP-biomedicals) en glucose by VETTEST 8008 dry chemical analyser.

2.4.4 Fish behavior

The effect of the experimental treatments was documented by behavioral observations using video cameras. Above each tank a camera was placed and connected to a video surveillance system (Sanyo DSR3716P). Camera focus was covering the entire tank surface but a representative part of it. Recordings took place on nine days from the 15th to the 31st of October 2007 between 11.00h and 11.10h to record undisturbed behavior and during 13.15h and 14.15h to document behavior during feeding time. Swimming behavior was measured by recording the number swim movements in one tank for 10 minutes defining a relocation of an individual for more than one body length was defines as movement. Results were related to the total number of fish per tank. If observations of the fish were impossible due to turbidity or foam on the tank surface, the records were discarded from analysis. Therefore in total datasets from 6 days for the smaller fish and 8 days for the bigger fish entered the analysis.

Feeding behavior was measured as latency to feed intake (time between the first contact between pellet and water and first consumption of pellets by the fish. Latency time was recorded by hand using a stop watch for 20 days (11th of February till 20th of May 2007).

2.5 Data analysis and statistics

2.5.1 Growth and feed intake

Growth was evaluated as weight development over time. Feed intake or better feed load was prior to analysis recalculated as feed load/ kg fish /day. The average individual fish weight per month was calculated as geometric average fish based on the sampling results of each month and tank. The weight data and the feed load were analyzed using *Generalized least estimates (GLS)* in R2.7.0 (R foundation, 2007/2008). This allows

comparing for differences between systems, accounting for the six weight groups and for difference over time. The used model was:

Model=gls(weight ~ factor(system) * days + factor(size) + RRKg + temp + correlation=corAR1(form=~1))
Systems is either RAS 1%, RAS 5% or flow through, days = days in the experimental period, size = class of the fish, RRKg = system water refreshment rate in m³/kg feed and temp = water temperature. Autocorrelation was corrected by using the corAR1 function. When one factor was insignificant (p>0.05) it was removed from the model and the model was run again. In total three datasets were subjected for analysis: 1) the whole experimental period, and the periods before and after reducing the system water refreshment rates (1-396d) and (396-579). This was done for the main classes S and L and as well for the six subclasses. Averaged weight gain (expressed as weight itself and specific growth rate, SGR) was tested as well for the start and the end of the experimental period using two-way ANOVA and defining SGR as:

$$\text{SGR (\%/dag)} = (\ln(W_t) - \ln(W_0)) * \frac{100}{T}$$

W_t and W_o as final and initial averaged individual fish weight and T as experimental time in days.

2.5.2   Physiology

Physiological data was recorded based on individual measurements. The data were averaged to obtain data per tank. Overall size classes S and L were tested using two-way ANOVA and the six smaller classes were tested using a one-way ANOVA in addition. Physiological data was as well subjected to analysis within the systems over size classes using a two-way ANOVA.
Condition factor and hematocrite was determined as:

$$Conditionfactor = 100 \times \frac{Weight}{Length^3}$$

$$\text{Hematocrite (\%)} = \frac{\text{height of bloodcells}}{(\text{Height bloodcells} + \text{height bloodplasma})} \times 100\%$$

Spleen weight was related to fish weight as % of body weight as spleen index.

2.5.3   Behaviour

Fish activity was analysed on tank level using one- and two-way and one-way ANOVA respectively to detect differences between systems integrating fish size classes. Latency to feed intake was tested using two-way ANOVA on log transformed data for differences between systems en size classes.

3   Results

3.1   Fish weight development and feed load

The weight development of the overall fish size is presented in Figure 4, Figure 5 and Table 4.
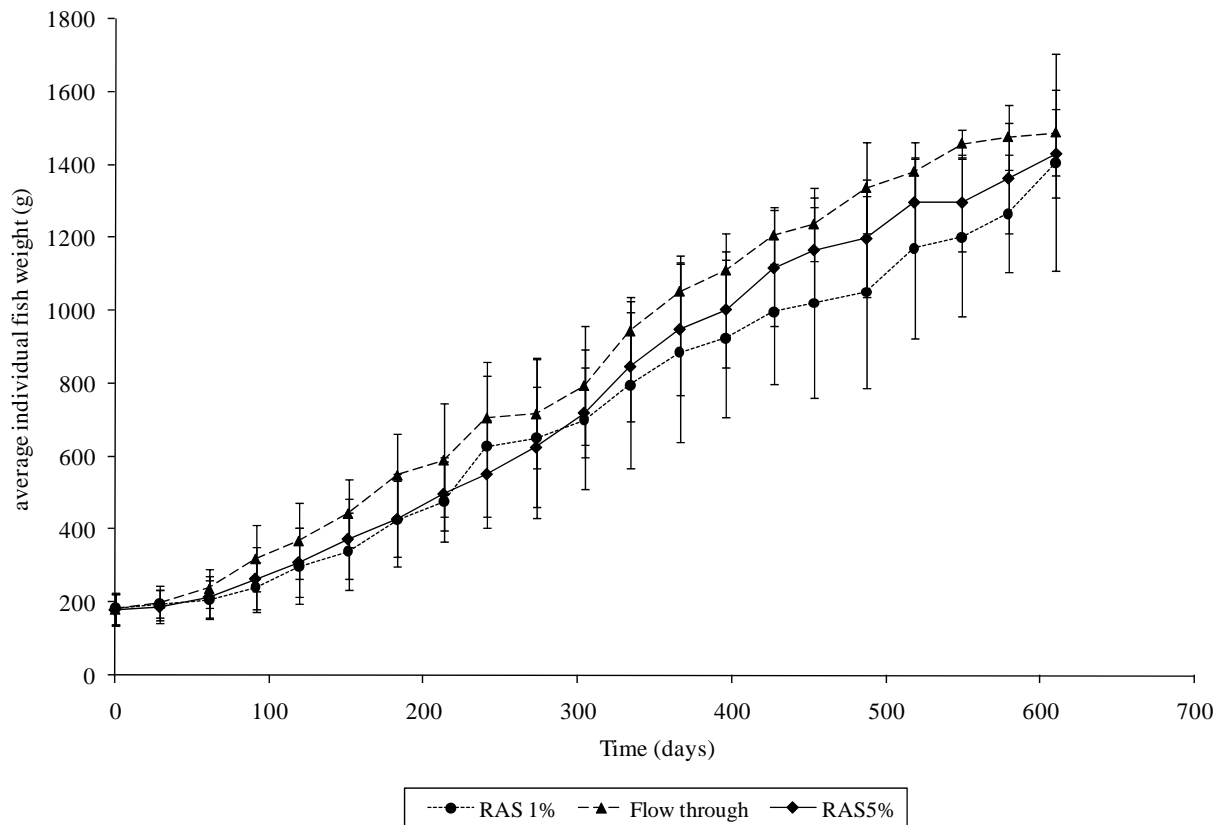

Figure 4: Weight development of the overall fish size class L, averaged for the three subclasses.
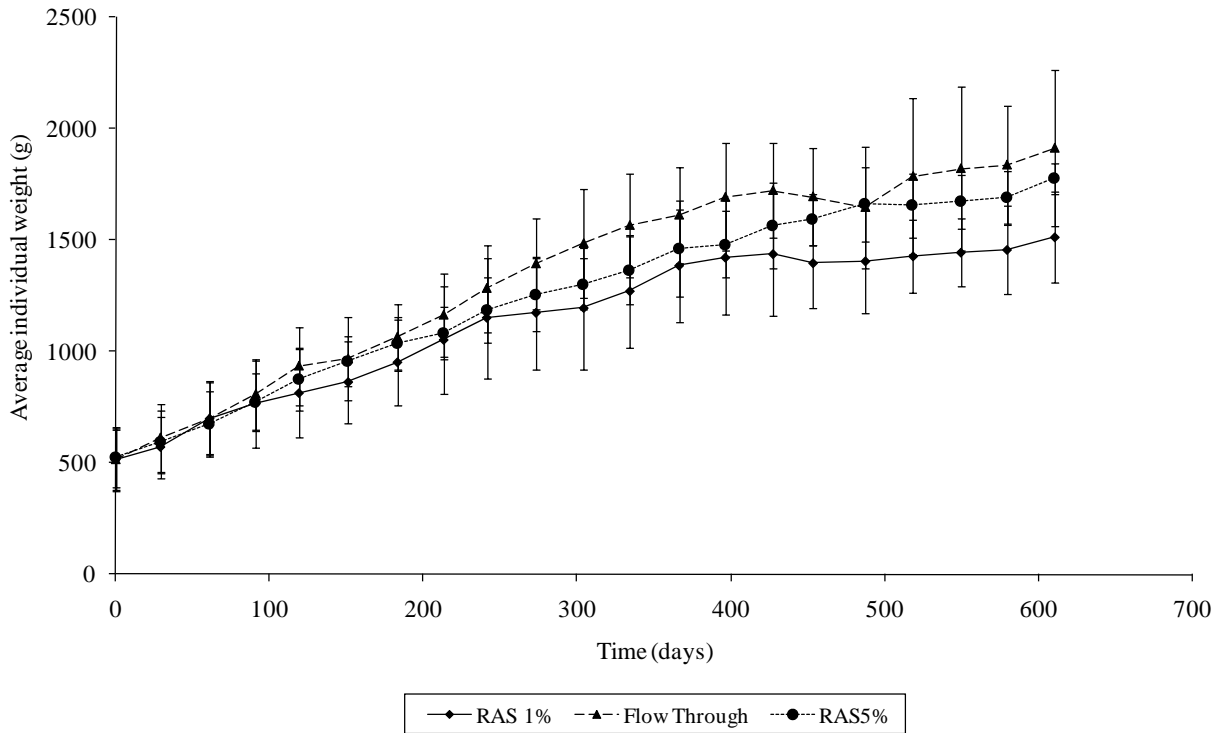
Figure 5: Weight development of the overall fish size class L, averaged for the three subclasses.

Table 4: Initial and final weight of the fish including all six size classes per system and their specific growth rate (SGR). Values in parenthesis are standard deviations)

System	Overall class	Size	Initial weight (g)	Final weight (g)		SGR (%/d)	
RAS 1%	L	S	375	1323		0.21	
		M	507	1490	1513 (203)	0.18	0.18(0.02)
		L	659	1727		0.16	
	S	S	143	1140		0.34	
		M	184	1353	1408 (229)	0.33	0.33 (0.01)
		L	229	1730		0.33	
Flow through	L	S	384	1512		0.22	
		M	504	2114	1914 (348)	0.23	0.22(0.02)
		L	654	2115		0.19	
	S	S	146	1354		0.37	
		M	175	1577	1489 (119)	0.36	0.35 (0.03)
		L	227	1535		0.31	
RAS 5%	L	S	401	1706		0.24	
		M	513	1778	1777 (203	0.20	0.20 (0.04)
		L	669	1846		0.17	
	S	S	138	1295		0.37	
		M	179	1472	1408 (229)	0.35	0.34 (0.03)
		L	226	1526		0.31	

P values for the comparison between systems were 0.27 and 0.20 respectively. Size class was significant with p>0.001 for SGR and 0.02 for weight. The interaction between system and size class had p values of 0.73 and 0.44 for SGR and weight respectively. The results from the GLS model showed majorly no differences for system itself but for the system days interaction as far as weight development is concerned. Feed load shows a different picture for the system influence with several significant p values and not for the system time interaction.

Table 5: p values derived from the gls model testing overall weight and feed load development accounting for size classes (day 0-549, day 0-396 and day 396-549) and for the different size classes using RRkg as system water refreshment rate (l/kg feed), temp as temperature(average per month) and the system time interaction.

	Intercept	RAS 1%	RAS 5%	days	RRkg	temp	RAS 1%*d	RAS 5%*d
Evaluated dataset	p-value	p-value	p-value	p-value	p-value	p-value	p-value	p-value
Weight								
Day 0-549	0.000	0.489	0.232	0.000	n.a.	0.000	0.000	0.056
Day 0-396	0.018	0.855	0.962	0.000	n.a.	n.a.	0.000	0.000
Day 396-549	0.000	0.729	0.402	0.000	n.a.	n.a.	0.065	0.874
SS	0.174	0.848	0.130	0.000	n.a.	n.a.	0.000	0.000
SM	0.000	0.824	0.660	0.000	n.a.	0.000	0.000	0.003
SL	0.000	0.035	0.006	0.000	n.a.	0.001	0.639	0.612
LS	0.240	0.100	0.028	0.000	n.a.	0.000	0.000	0.435
LM	0.000	0.945	0.751	0.000	n.a.	n.a.	0.000	0.000
LL	0.000	0.079	0.083	0.000	n.a.	n.a.	0.000	0.002
Feed load								
Day 0-549	0.000	0.000	0.000	0.000	0.000	0.000	0.373	0.945
Day 0-396	0.004	0.000	0.000	0.008	0.000	0.000	0.715	0.434
Day 396-549	0.013	0.032	0.084	0.440	0.033	n.a.	0.080	0.255
SS	0.002	0.006	0.018	0.017	0.001	0.006	0.947	0.994
SM	0.229	0.050	0.050	0.027	0.040	0.000	0.104	0.700
SL	0.396	0.071	0.406	0.094	n.a.	0.011	0.778	0.432
LS	0.609	0.001	0.325	0.224	n.a.	0.010	0.003	0.029
LM	0.000	0.006	0.001	0.001	0.003	0.038	0.717	0.192
LL	0.013	0.028	0.031	0.011	0.005	0.000	0.437	0.505

3.2 Physiology

The physiological parameters were recorded on day 258 and day 552 of the experiment. There was no difference for hematocrite values for the first sampling between systems including the analysis for different size classes (Table 6). Higher hematocrite values were observed for the smaller turbots compared to the larger fish, irrespective of system. During the second sampling RAS 1% showed higher hematocrite values compared to the other systems. However no effect was recorded for the size classes separated. During the second sampling the larger turbots showed higher hematocrite values than the smaller turbots, in contrast to the first sampling ($p<0.01$). The values between the first and the second sampling differed for the larger fish, showing higher values during the second sampling ($p=0.01$)

Table 6: Physiological parameters measured on day 258 (first sampling) and on day 552 (second sampling). Values are average values and standard deviations in parenthesis per system with n = 6 tanks/system, n = 5 or 10 fish/tank) and per size class (n = 9 tanks/size class S or L, n =5 or 10 fish/tank). Values with different superscripts differ significantly (p<0.05) by system or size class (Two-way ANOVA). Least square differences (LSD) are only given for significant different values.

	Hematocrite	Spleen Index	Cortisol	Glucose	Condition factor
	(%)	(%)	(ng/ml)	(mmol/L)	
First Sampling					
System					
Flow Through	21.0 (1.7)	0.13 (0.01)[a]	5.30 (8.6)	2.08 (0.32)[a]	1.78 (0.10)
RAS5%	20.2 (2.7)	0.10 (0.02)[b]	12.7 (13.6)	2.33 (0.15)[a]	1.76 (0.08)
RAS1%	19.4 (2.2)	0.13 (0.02)[a]	7.4 (8.3)	1.72 (0.21)[b]	1.76 (0.10)
Overall Size class					
S	21.4 (1.3)[a]	0.12 (0.03)	12.5 (12.1)	2.02 (0.31)	1.72 (0.06)[a]
L	19.0 (2.4)[b]	0.11 (0.02)	4.4 (6.6)	2.06 (0.36)	1.81 (0.09)[b]
P values					
System	N.S.	0.029	N.S.	< 0.001	N.S.
Size class	0.012	N.S.	N.S.	N.S.	0.030
System * Size class	N.S.	N.S.	N.S.	0.016	N.S.
LSD. (5%)					
System	-	0.02	-	0.2185	-
Size class	0.017	-	-	-	0.079
System * Size class	-	-	-	**0.3090**	-
Second sampling					
System					
Flow Through	18.9 (0.9)[a]	0.14 (0.02)[a]	2.76 (2.8)	1.82 (0.17)	1.90 (0.08)[a]
RAS5%	18.1 (1.0)[a]	0.17 (0.02)[b]	3.42 (4.9)	1.88 (0.17)	1.80 (0.08)[b]
RAS1%	19.7 (1.4)[b]	0.15 (0.02)[ab]	0.91 (1.2)	1.78 (0.15)	1.76 (0.07)[b]
Overall Size class					
S	18.4 (1.2)[a]	0.15 (0.03)	2.42 (2.6)	1.88 (0.18)	1.77 (0.06)[a]
L	19.4 (1.2)[b]	0.15 (0.02)	2.31 (4.0)	1.77 (0.12)	1.87 (0.10)[b]
P values					
System	0.07	0.10	N.S.	N.S.	< 0.01
Size class	0.10	N.S.	N.S.	N.S.	< 0.01
System * Size class	N.S.	N.S.	N.S.	N.S.	N.S.
	(P<0.10)	(P<0.10)			(P < 0.05)
LSD	1.14	0.024	-	-	0.07
System	0.93	-	-	-	0.06
Size class	-	-	-	-	

The spleen index was lowest in RAS 5% during the first sampling and similar in the other systems (Table 7). No effect of size class on spleen index was observed during the first sampling (Table 6). Despite this, the system effect on spleen index seems attributable to the small fish (S), as within the size class L no differences between systems were observed while for size class S a lower spleen index was observed in RAS5% compared to RAS1% (Table 7). The spleen index observed in the flow through system was not different from both RAS1% and RAS5% for both size class S and L.

The results of the second sampling are in contrast to the results of the first sampling. A trend (P<0.10) towards a higher spleen index in RAS 5% compared to flow through was observed, while RAS1% did not differ from the

other systems (Table 6). Again no size class effect on spleen index was observed (Table 6), while a trend towards a system effect on spleen index was observed within size class L., and, in contrast to the first sampling, no system effect was observed within size class S (Table 7).

Overall spleen index between the sampling events changed significantly (P<0.001) and measured values were higher compared to the first sample event. For the small fish there was no effect detected between sample events within RAS 1% (lower water refreshment rate) and for the larger fish a trend (p=0.09) was found.

During first sampling, glucose concentrations in blood plasma were significantly lower in fish of RAS 1% for the larger fish and similar for the flow through and RAS 1% but significant lower than RAS 5%. During second sampling no differences in glucose concentration in the blood plasma were found between systems and size classes (Table 6). Also within size classes no system effect on glucose was observed for both size classes (Table 7). Lower glucose concentrations in the blood plasma were found on the second sampling day for the large turbot in flow through and for the small turbot in RAS5% compared to sampling day 1.

The average cortisol concentrations in the blood plasma showed a high variation within systems due the underlying individual variation. No differences in cortisol levels were found between systems and size classes on both sampling days (Table 6 and 7).

Lactate concentrations in the blood plasma were below the detection limit of 0.50 mmol/L in all cases.

The condition factor was not significant different during the first sample event. Independent of the system effect, condition factor was higher in larger fish. During the second sample event there was an effect of both system and size class on condition factor (table 6) with lower values for the two RAS's for smaller and larger fish respectively (Table 7). In the flow through system the condition factor was higher for both size classes on the second sampling day compared to the first sampling day (Table 7).

Table 7: Physiological parameters measured on 258d and 552d (first and second sampling, L=large and S=small). Values are average values and standard deviations in parenthesis per system with n = 6 tanks/system, n = 5 or 10 fish/tank) and per size class (n = 9 tanks/size class S or L, n =5 or 10 fish/tank). Values with different superscripts differ significantly (p<0.05) by system or size class (one-way ANOVA). Least square differences (LSD) are only given for significant different values. P-values for differences between the first and second sampling day within systems result from One way ANOVA with sampling day as factor and indicate if average values per size class and system were differed between sampling days.

	Hematocrite (%)		Spleen Index (%)		Cortisol (ng/ml)		Glucose (mmol/l)		Condition Factor	
Size class	L	S	L	S	L	S	L	S	L	S
First Sampling										
Flow Through	21.2 (2.7)	20.9 (0.6)	0.12 (0.02)	0.13 (0.01)[ab]	0.3 (0.52)	10.3 (10.4)	2.30 (0.14)[a]	1.86 (0.17)[a]	1.86 (0.05)	1.70 (0.03)
RAS5%	18.3 (1.7)	22.1 (2.1)	0.11 (0.02)	0.10 (0.02)[b]	9.9 (9.8)	15.5 (18.6)	2.25 (0.15)[a]	2.40 (0.13)[b]	1.77 (0.09)	1.76 (0.08)
RAS1%	17.6 (1.4)	21.2 (0.7)	0.11 (0.01)	0.14 (0.02)[a]	2.9 (2.74)	11.8 (10.3)	1.64 (0.26)[b]	1.81 (0.16)[a]	1.81 (0.13)	1.71 (0.04)
P values	0.14	0.54	0.34	0.05	0.20	0.89	0.01	< 0.01	0.53	0.35
LSD	-	-	-	0.037	-	-	0.39	0.30	-	-
Second Sampling										
Flow through	19.3 (0.9)	18.4 (0.7)	0.13 (0.01)	0.15 (0.03)	2.63 (2.5)	2.90 (3.7)	1.90 (0.21)	1.74 (0.09)	1.96 (0.04)[a]	1.83 (0.06)[a]
RAS5%	18.5 (1.1)	17.7 (0.9)	0.16 (0.00)	0.18 (0.04)	3.10 (4.2)	3.73 (6.5)	1.93 (0.19)	1.84 (0.18)	1.87 (0.03)[ab]	1.74 (0.03)[b]
RAS1%	20.2 (1.3)	19.2 (1.5)	0.15 (0.02)	0.14 (0.02)	1.53 (1.4)	0.29 (0.5)	1.82 (0.21)	1.74 (0.08)	1.78 (0.10)[b]	1.74 (0.01)[b]
P value	0.26	0.29	0.07	0.40	0.86	0.62	0.80	0.56	0.04	0.05
LSD	-	-	-	-	-	-	-	-	**0.13**	**0.08**
P-values First vs. Second sampling										
Flow through	0.32	0.01	0.53	0.46	0.19	0.31	0.05	0.32	**0.05**	**0.03**
RAS5%	0.87	0.03	0.004	0.03	0.33	0.36	0.09	0.01	**0.13**	**0.65**
RAS1%	0.08	0.10	0.02	0.90	0.46	0.19	0.41	0.56	**0.70**	**0.52**

3.3 Behaviour

The average swimming activity was lower in RAS 1% compared to the other two systems (Table 8). Furthermore a trend was observed ($p<0.10$) for larger fish towards higher activities. For the smaller fish size classes there was no difference between the systems (Figure 6). For the larger fish the activity was lower in RAS 1% compared to the flow through system and RAS 5% (Figure 7).

Table 8: Swimming activity and latency to feed uptake using averaged values per system (activity, n=42) and averaged values per size class (L n=24 and S n=18) Different superscripts are illustrating significant differences (p<0.05)

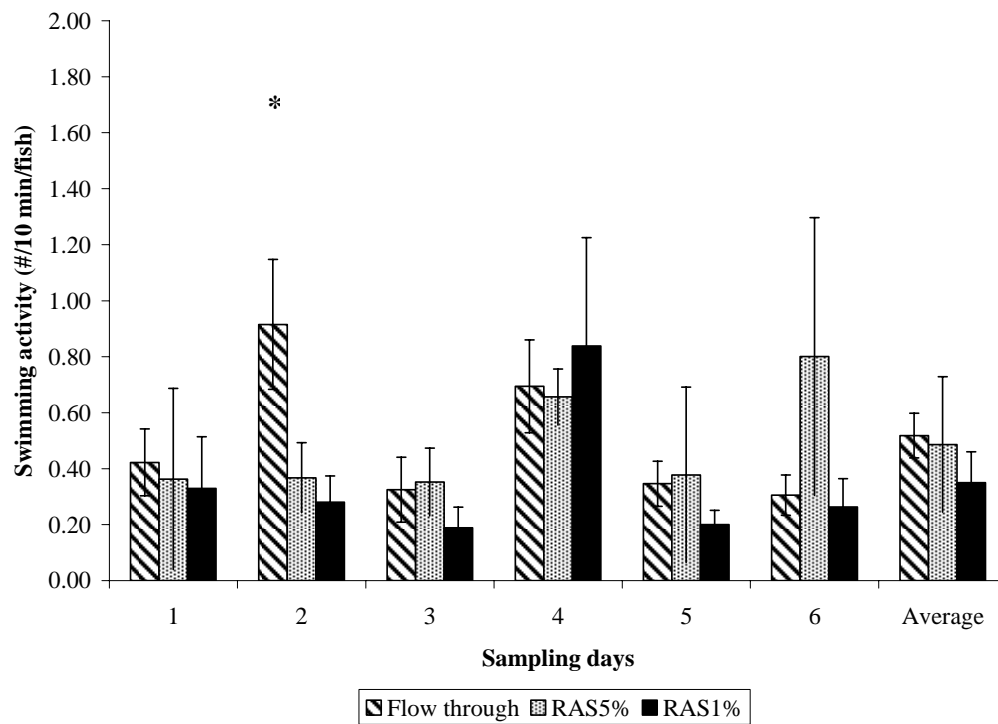|	Activity	Latency time till feed intake
	(# Swimming movements/fish/10min)	(sec)
System		
Flow through	0.57[a]	1.87 (0.43)
RAS5%	0.64[a]	2.37 (0.25)
RAS1%	0.34[b]	2.19 (0.22)
Size Class		
Small	0.45	2.09 (0.47)
Large	0.58	2.20 (0.23)
P values		
System	0.007	< 0.001
Size class	0.08	N.S.
System * size class	N.S.	0.005
L.S.D. (5%)		(log LSD)
System	0.17	0.05235
Size class	-	-
System * size class	**-**	**0.04274**

Figure 6: Average (n = 3) swimming activity per sample day and the average over sample days per culture system for the smaller turbots. Values with asterix differ significantly (p<0.05)
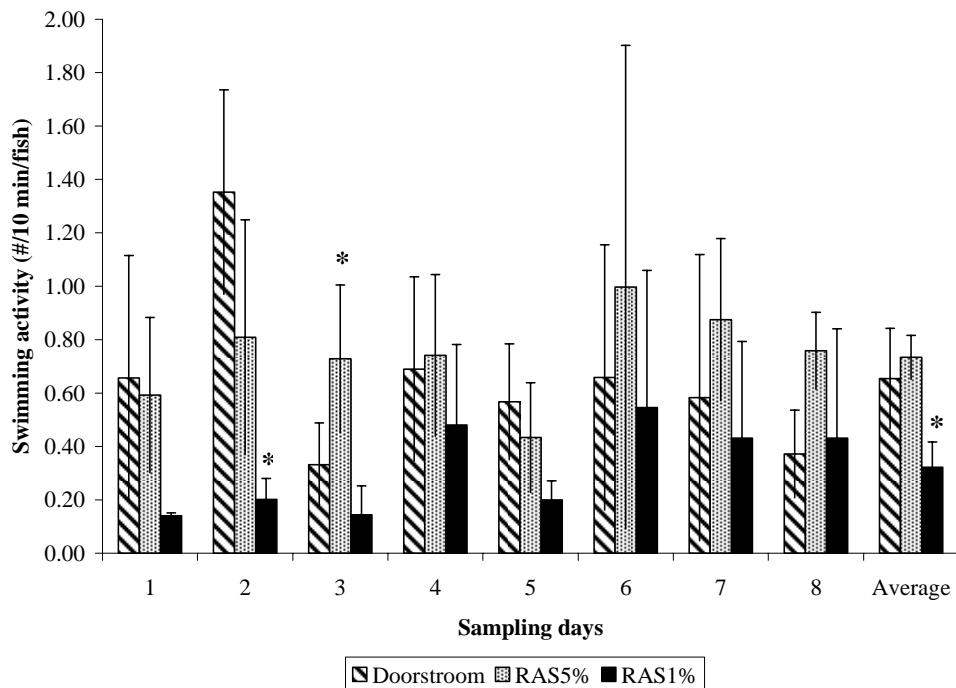

Figure 7: Average (n = 3) swimming activity per sample day and the average over sample days per culture system for the larger turbots. Values with asterix differ significantly (p<0.05)

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The averaged latency to feed intake was significant shorter in the flow through system (Table 8). Within the flow through system there was a shorter latency time recorded in the smaller fish compared to the other size classes (Figure 8)
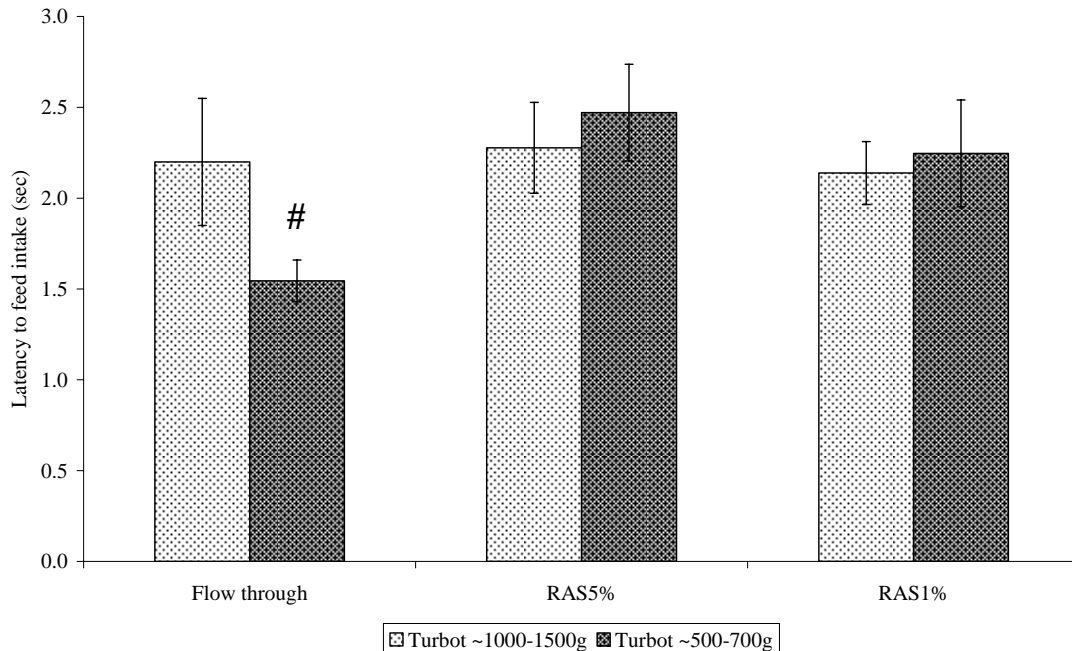

Figure 8: Average latency time to feed intake for the three culture systems and two size classes. # indicates significant differences (p<0.05).

3.4   Waterquality

Water quality was in the range widely accepted for Turbot culture. Important to note are differences in temperature which have been included in the weight development and the feed intake models. All other values are not suspected to influence fish growth or feed intake and are therefore not discussed.

Table 9: Water quality recorded during the experiment by system as average and standard deviation (SD) and the range noticed.

Parameter	Dimension	Flow through	RAS5%	RAS1%
Oxygen saturation	(%)			
Average		168 (2.8)	176 (2.3)	175 (2.1)
Range		156 - 181	147 - 198	150 - 198
Temperature	(°C)			
Average		14.6 (0.8)	15.4 (2.0)	15.1 (2.4)
Range		13.5 - 16.5	12.5 – 18.0	11.5-18.3
pH				
Range		7.53 – 7.76	7.43 – 7.79	7.20 – 7.83
Total ammonia	(mg/L)			
Nitrogen				
Average		0.52 (0.17)	0.56 (0.19)	0.71 (0.28)
Range		0.25 – 0.85	0.25 – 0.97	0.35 – 1.38
Nitrite	(mg N/L)			
Average		0.08 (0.07)	0.22 (0.16)	0.30 (0.10)
Range		0.02-0.19	0.05 – 0.69	0.12 – 0.45

4 Discussion

4.1 Fish weight development and feed intake

The results from the statistical analysis showed no differences for weight development influenced by system itself but for the system * day interaction. Water temperature is influencing weight development, which can be expected based on simple rules of bioenergetics. The strong interaction effect between system and days hints towards the fact that the sampling, thus the generation of sample values over time has influenced the slope of the curve. This would mean that the commercial way of handling and grading the fish that was applied in the experiment influenced the data more than the overall system effect did. That means, even if there is any effect of system it is entirely overruled by the sampling procedure. The p values that are smaller than 0.05, which were detected for two size classes, cannot be considered to influence this overall conclusion, as they are prominent for the RAS with the highest refreshment rate and should be most prominent for the system with the lowest one (RAS 1%) to support the hypothesis of lower growth in more closed RAS. The obtained values and comparisons for initial and final weights and for SGR support this hypothesis further. The overall growth that was obtained in the system is comparable to data obtained on commercial farms but lower than values obtained in several farms and in laboratory experiments. This illustrates the value and importance to conduct such experiments in a research environment. The differences and lower levels might be explained by several factors, such as the grade and family of the fish and most of all the lower water temperatures compared to other commercial units. Dutch RAS systems are normally running on temperatures close to $20^{o}C$, which was not realized in the facility used. The lower feed intake but similar growth in the two RAS might be due to the fact that in RAS systems more stable microbial environments are established that will not impact fish welfare as much as in flow through systems. It can be further speculated that the fluctuating conditions in flow through system require more adaptation and therefore more energy has to allocated to other goals than growth by the fish. It is longer hypothesized that growth in well managed RAS should be better than in flow through systems due to the more stable biotic as well as abiotic conditions. This should lead to higher feed efficiency. For feed intake furthermore a system effect was observed, which supports this hypothesis.

4.2 Physiological parameters

Hematocrite in turbot varies between 11 to 15% (Pichavant *et al.*, 2002; Person-Le Ruyet *et al.*, 2002; Cal *et al.*, 2005; Quentel en Obach, 1992). Measured values are all inside this range. Even the obtained differences in the second experiment do only relate to small differences and not to strong system effects. Increase glucose concentrations can hint on stress experience in fish. Normal values are between 2 and 3 mmol/l and acute stress can lead to values of 3-4mmol/l (Van Ham *et* al., 2003). The here measured values are all in the range for non stressed turbots, whereby RAS 1% had the lowest values. This might be related to the lower swimming activity in that system.
Spleen index was found to be affected by system and size class (Tables 6 and 7). However the results are not consistent on sampling days and between sampling days. For example during first sampling, the observed system effect on spleen index observed in size class S conflicts with the absence of an overall size class effect on spleen index. This shows that the observed systems effect on spleen index is small, which is further supported by the observation that the system with the lowest spleen index during first sampling, yielded the highest spleen index during second sampling. Changes in spleen size hint often to diseases or immunological issues (Goede en Barton, 1990). The splenosomatic index (spleen index) is therefore an indicator for animal health status (Hutchinson en Manning, 1996). A spleen index of 0.05% has been proposed as a representative value for turbot (Quentel en Obach, 1992), which is much lower than the currently observed values ranging from 0.10% to 0.17% for the three systems. This means that the immune system of all turbots in the experiment was more active than expected based on literature. However, since leucocrite could not be detected in all cases it is highly unlikely that sampled fishes suffered from chronic stress or diseases. Also the results for the condition factor are inconclusive and do not support the hypothesis that there is any difference between the system management styles.
Cortisol levels in the blood plasma were equal in all cases. The large standard deviations of mean values resulted from the high underlying individual variation in cortisol levels, which can be attributed to individual variation in stress response (Costas, 2008). For this reason the number of sampled turbot was increased from five to ten turbot per tank during the second sampling day. Indeed lower but still relatively high standard deviations of mean

cortisol levels found. Therefore it cannot be excluded that small system effects on cortisol levels existed but could not be demonstrated. On the other hand the current results demonstrate that culture systems did not result in large differences in cortisol levels. In addition the data clearly show that none of the culture systems result in acute stress among the sampled turbot as mean values did not exceed 15.5 ng/mL, while acute stress in turbot results in blood plasma cortisol levels of 50 to 80 ng/mL (Van Ham *et al.*, 2003).

4.3   Behavior

The average swimming activity of the large turbot was lower in RAS1% compared to Flow through and RAS5% (Fig 7). However, of the eight individual samplings days only sampling day 2 and 3 showed significant differences among systems for the large fish. For the small fish no differences in the average swimming activity were observed among treatments, while the swimming activity on the individual sampling days showed significant differences on only one sampling day (Fig 6). The meaning of these observations and the relation (if any) to the culture systems remain unclear. Comparison to turbot activity in natural or captive conditions is not possible since information is lacking.
Turbot showed a higher feeding motivation (responded faster to feed administration) in Flow through compared to RAS1% and RAS5%, and this observation can be attributed to the large turbot (Fig 8). We also found that in feed load was higher in Flow through compared to RAS (Table 5) but this did not result in higher growth (Table 5). Therefore it remains unclear if feeding motivation and feed intake are related and in what way.

5   Conclusions

It can be concluded based on this elaborated experiment that the hypothesis that changes in feed intake can be associated with changed fish welfare status in fish, using turbot as model species cannot be supported if closed RAS system were assumed to impair fish welfare status. In contrary feed intake in RAS 1% and RAS 5% was significantly influenced by system and lower than in the flow through system. The second hypothesis that changed fish welfare status is caused by different system water refreshment rates and fish and system management, leading to lower growth rates and less feed intake in more closed RAS, can therefore neither be supported. All the obtained differences in feed intake however are not reflected in the physiological or behavioral data hinting in the direction that fish welfare is not impaired in either system type.

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The scientific quality of this report has been peer reviewed by the a colleague scientist and the head of the department of Wageningen IMARES.

Approved: Henk van der Mheen
                             Head Department of Aquaculture

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