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Reducing geosmin off-flavor compounds and waste outputs through dietary phosphorus management in rainbow trout aquaculture

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ABSTRACT: The aquaculture industry has long recognized the need to reduce phosphorus (P) waste outputs associated with environmental impact, and reduce off-flavor producing compounds, which can impact the quality of the fish product. This study was undertaken to investigate the effects of dietary high P (HP) and low P (LP) on growth, nutrient digestibility, P retention, and P loading as well as their correlation to the synthesis of geosmin-associated off-flavor in a recirculating aquaculture system of rainbow trout *Oncorhynchus mykiss*. The above diets were fed to quadruplicate tanks of rainbow trout (average mass ± SD: 127.4 ± 3.1 g) for 84 d. Results showed that the effects of the HP and LP diets on growth and P retention were not significantly different. While the apparent digestibility of P and other nutrients were higher in fish fed the LP diet, P waste outputs and geosmin levels in the fillets of fish were higher in fish fed the HP diet. Magnesium (Mg^{2+}), potassium (K^{+}) and zinc (Zn^{2+}) concentrations in tank water were significantly lower in fish fed the HP diet than the LP diet in most of the sampling events. Furthermore, the tank water geosmin concentration was not strongly proportionally correlated with tank water-soluble P concentration for both the LP and HP diets. There was a strong proportional linear relationship between the geosmin concentration in tank water and in trout fillet for both the LP and HP diets. Results suggest that off-flavor contents in fish fillets and water were related to the dietary P level and metabolic P waste outputs into the system, findings that have implications for the formulation of sustainable diets for rainbow trout.

KEY WORDS: Recirculating aquaculture system · Metabolic phosphorus waste · Off-flavor · Geosmin · *Oncorhynchus mykiss* · Growth · Nutrient digestibility · Nutrient retention

INTRODUCTION

The presence of off-flavors in fish raised in recirculating aquaculture systems (RAS) represents one of the most significant economic problems related to product quality encountered in aquaculture. The presence of undesirable odors or tastes in fish may cause a major reduction in human consumption of the products, or make them unsuitable for sale. Among those flavors, the 'earthy' and 'muddy' odors constitute >80% of the off-flavor problems found in farm-raised catfish (Grimm et al. 2000). Such off-flavors come from the absorption by fish of substances including geosmin and 2-methylisoborneol (2-MIB), which are produced by a broad group of aquatic microorganisms (Gerber 1969, Tucker 2000, Bai et al. 2013). In our previous RAS studies, negligible MIB concentrations were found in fillets of arctic charr and rainbow trout; these studies also clearly revealed that off-flavor was caused mainly by geosmin (Auffret et al. 2011, Houle et al. 2011).

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Elevated phosphorus (P) levels in freshwater aquaculture effluents may lead to environmental problems (Schindler 1977, Camargo & Alonso 2006), as intensive aquaculture can generate environmental P loading that may contribute to the eutrophication of sensitive receiving water bodies (Lall 1991, Sarker et al. 2013). Furthermore, it is often claimed that eutrophic waters are the most vulnerable to off-odors. The environmental factors known to lead to high levels of geosmin by producing nuisance blooms of some microorganisms, such as streptomycetes, myxobacteria and cyanobacteria, include high nutrient concentrations and ratios (Smith & Bennett 1999, Downing et al. 2001, Robin et al. 2006, Schrader & Summerfelt 2010).

A number of investigators have reported that P concentration correlates with geosmin concentration (Saadoun et al. 2001, Robertson et al. 2006, Robin et al. 2006, Dzialowski et al. 2009). Since the majority of P released by aquaculture operations is ultimately from dietary origin, effective management of waste outputs can be achieved through management of the nutrient composition of feeds. However, the literature lacks data on whether dietary P and the consequential metabolic wastes can induce geosmin-associated off-flavor in RAS-raised rainbow trout. Our recent preliminary studies demonstrated that geosmin-associated off-flavor in rainbow trout correlates to elevated P levels in feed, resulting in the excretion of excess P into the water (authors' unpubl.). We hypothesize that a high-P trout feed may induce geosmin-associated off-flavor in rainbow trout raised in RAS. The purpose of the current study is to investigate the effects of dietary P levels on the synthesis of geosmin in water and trout fillet. In particular, our goal is to evaluate a low-P (LP) diet containing 5.4 g kg^{-1} total P and a high-P (HP) diet containing 13.0 g kg^{-1} P using the response criteria of weight gain, nutrient digestibility, P retention, P waste outputs and geosmin concentrations in trout fillet and water.

MATERIALS AND METHODS

Experimental diets, fish rearing, feeding and design

Two iso-nitrogenous and iso-energetic diets were formulated, all based on the same basic feed containing balanced levels of essential amino acids, fatty acids, vitamins and minerals, except for P and calcium (Ca) levels (Table 1). The 2 practical diets contained 5.4 and 13.0 g kg^{-1} total P (LP and HP, respectively). The HP is closest to that of standard fish feed for rainbow trout. The HP diet was obtained by supplementing 3.5% inorganic phosphorus salt (i.e. 35 g $CaHPO_4$ kg^{-1}) in the basal diet. Rainbow trout *Oncorhynchus mykiss* (initial average mass: 127.4 ± 3.1 g) was used. The experimental set-up was arranged in a completely randomized design; each diet was fed to 4 replicate rectangular tanks (380 l volume), each containing 43 fish. The feeding experiment was conducted in an RAS

Table 1. *Oncorhynchus mykiss*. Experimental diet formulation (as fed basis) and chemical composition (dry matter basis). Vitamin premix and mineral premix: supplied the following: as per NRC (1993) (to provide mg kg^{-1} except as noted): retinyl acetate, 2500 IU; cholecalciferol, 2400 IU; tocopheryl acetate, 50; menadione, 10; thiamin, 1; riboflavin, 4; pyridoxine, 3; Ca-pantothenate, 20; vitamin B-12, 0.01; niacin, 10; biotin, 0.15; folic acid, 1; choline, 1000; inositol, 300; magnesium carbonate, 1.24 g; calcium carbonate, 2.15 g; potassium chloride, 0.90 g; sodium chloride, 0.40 g; potassium iodide, 0.4; copper sulfate, 30; cobalt sulfate, 0.2; ferric sulfate, 0.20 g; manganese sulfate, 30; zinc sulfate, 40; sodium fluoride, 10. Digestible phosphorus (P) = Total P in the diet × P digestibility/100

	Diet		Canadian supplier
	Low P	High P	
Ingredients (g kg^{-1})			
Herring meal	75	75	SANIMAX
Blood meal (non-ruminant; APC Inc. AP 301)	100	100	SANIMAX
Feather meal	100	100	Floradale Feed Mill
Wheat grain	205	180	Meunerie Gérard Soucy
Soybean meal (46%)	80	80	Meunerie Gérard Soucy
Wheat gluten	70	70	ADM Alliance Nutrition
Corn gluten meal	170	170	Meunerie Gérard Soucy
Fish oil	180	180	SANIMAX
$CaHPO_4$ (Laboratoire mat, CR-0118)	0	35	Meunerie Gérard Soucy
Lysine (Biolys)	12	12	SANIMAX
DL-methionine	2	2	Corey Feed Mills
Vitamin premix and mineral premix	6	6	Corey Feed Mills
Antioxidant (ppm) (ethoxiquin 0.015%)	100	100	Corey Feed Mills
Proximate composition (% as dry matter)			
Dry matter	90.45	90.18	
Crude protein	49.71.	49.93	
Lipid	20.79	21.39	
Energy (MJ kg^{-1})	24.93	24.43	
Total phosphorus	0.54	1.30	
Digestible phosphorus	0.32	0.69	
Ash	4.16	7.11	

system at the Laboratoire Régional des Sciences Aquatiques (LARSA), Université Laval. The recirculation rate was >90%. The flow of makeup water, which was from a municipal source, dechlorinated and filtered (45 µm), was fixed at 20 l h^{-1} for a retention time of 3.5 d. Oxygen concentration (90–100% saturation) and water temperature (15.0°C) were monitored in real-time throughout the experiment. The pH (7.4) was measured once daily and sodium carbonate was added to maintain pH above neutral.

Prior to the beginning of the growth trial, fish were acclimated for 2 wk with the LP diet. Tank by tank basis pair-feeding was employed to supply the same quantity of dietary nutrients (feed) to the groups, but P supply was variable, as the dietary P content of the 2 diets differed. LP replicates were hand-fed until apparent satiation 2 times a day, and the same quantity was fed to HP replicates. The experimental design and protocols were approved by the Canadian Council on Animal Care (CCAC 1984) and the Animal Protection Committee of Université Laval (CPAUL 2010).

For the digestibility measurement of the diet, 1% Sipernat 50™ (a source of acid-insoluble ash [AIA]) was added to the diet as an indigestible marker. Microingredients were first mixed and then slowly added to the macroingredients to ensure a homogeneous mixture. The ingredients were thoroughly mixed and steam-pelleted using a California Pellet Mill, and pellets were dried in a forced-air oven (22°C, 24 h), sieved and stored at −20°C.

Growth measurement and fish fillet preparation

Fish were bulk-weighed at the beginning of the experiment, and then every 3 wk until the end of the experiment (84 d). At the start of the experiment, 5 fish were sampled from the initial population; after which 3 fish per tank were sampled every 21 d (Days 0, 21, 42, 63 and 84). The effects on growth were determined by evaluating gain in fish mass, total feed consumption, specific growth rate (SGR), feed conversion ratio (FCR) and thermal-unit growth coefficient (TGC). Indices were calculated as follows:

$$\text{Gain in fish mass} = [(\text{Final body mass} - \text{Initial body mass}) / \text{Initial body mass}] \times 100\% \quad (1)$$

$$\text{FCR} = \text{Feed intake} / \text{Body mass gain} \quad (2)$$

$$\text{SGR} = 100 \times (\ln \text{Final body mass} - \ln \text{Initial body mass}) / \text{Days} \quad (3)$$

$$\text{TGC} = 100 \times (\text{Final mass}^{1/3} - \text{Initial mass}^{1/3}) / \text{Sum of daily water temperature} \quad (4)$$

At each sampling event, length and weight were determined, fish were immediately filleted and the skin removed. Two fillets per fish were stored in a Ziploc bag at −20°C for further geosmin (using solid phase micro extraction [SPME] with GC-MS) and lipid analysis.

Water sample collection

At every sampling time point, 3 water samples were taken in each tank from 3 different places. Individual water samples were stored in 20 ml glass scintillation vials, 6 g of NaCl and a magnetic stir bar were then added just prior to analysis. The vials were filled completely, as indicated by the fact that no air bubbles were observed when the vial was capped and inverted, and were then stored at 4°C for further geosmin analysis. Geosmin concentration was analyzed the day after the sampling to avoid any reduction of geosmin content of the samples. At every sampling time point, 3 water samples were collected from different places of each sand filter, biofilter and pump using 20 ml glass scintillation vials, and were stored at 4°C for further geosmin analysis. To determine the concentration of soluble reactive phosphorus, anions and cations that had accumulated in the tank, sand filter, biofilter and pump water filter, water samples were collected in triplicate into a disposable 15 ml falcon tube (containing 2 mM HCl) (Falcon®, Becton Dickinson) on Days 0, 21, 42, 63 and 84. Samples were stored at 4°C for further analysis.

Fecal collection

Feces were collected at the end of the feeding trial once a day for 2 wk via a modified Guelph system based on Cho et al. (1982) before the morning meal. Tanks were brushed and purged immediately and any uneaten feed residues and feces were flushed out of the fecal collection column after each feeding. The feces were decanted, excess water was removed and then the feces were stored at −20°C until the end of the 2 wk of feces collection. Feces were freeze-dried for 7 d prior to analysis to determine apparent digestibility coefficients (ADCs) for the nutrients.

Diet and feces chemical composition

A pool of different feed sizes was collected for analysis of the feeds. Dry matter, ash, crude pro-

tein, total lipid, and gross energy in feed and feces were analyzed according to standard procedures (AOAC 1990). Fillet dry matter was obtained by first drying the pools in a lyophilizer for 7 d, and then in a forced-air oven at 105°C overnight. Sample weight was recorded before and after drying, followed by cooling in a desiccator (AOAC 1990). Feed and feces dry matter was obtained by drying only in the forced-air oven. Ash content was obtained by dry-ashing in porcelain crucibles in a furnace at 500°C overnight and expressed as dry weight (AOAC 1990). Gross energy was performed by bomb calorimetry (Parr Instrument Company) and calculated as percentage of dry matter. Crude protein was evaluated using a LECO analyzer (model FP-2000), then a nitrogen (N) conversion factor of N × 6.25 was used, with crude protein expressed as dry weight.

Total lipid composition was performed with an Ankom lipid extraction instrument (XT-10, Ankom Technologies), the solvent being diethyl ether. Total lipids were expressed as dry weight. To determine the phosphorus concentration, acid digestion with 5 ml concentrated HNO_3 and 12.5 ml HCl (50%) solution was used, and then resulting materials were filtered (Whatman paper No. 1) and then measured using an ion chromatography system (Dionex, ICS-3000).

Nutrient (P, N and energy) retention was calculated as:

Nutrient retention (g kg^{-1} fish) = 100 × [(Final biomass × Final nutrient concentration of the fish) – (Initial biomass × Initial nutrient concentration of the fish)] / Feed consumed × Nutrient concentration of the diet (5)

Total P loading was estimated based on solid and dissolved P loading (solid P load + dissolved P load). Solid and dissolved nutrient (P, N and energy) load was calculated as follows:

Solid nutrient load (g kg^{-1} fish) = [1 – Apparent nutrient digestibility coefficient × Nutrient intake (g kg^{-1} fish)] (6)

Dissolved nutrient load (g kg^{-1} fish) = {Apparent nutrient digestibility coefficient × [Nutrient intake (g kg^{-1} fish) – Retained nutrient (g kg^{-1} fish)]} (7)

AIA in diets and feces were determined according to the official methods described by Keulen & Young (1977). The ADCs of P were calculated as described by Cho & Slinger (1979) using the following formula:

ADC = 100 × [1 – (% Nutrient of feces / % Nutrient of diet) × (% Marker in diet / % Marker in feces)] (8)

Geosmin assay

The method of Lloyd & Grimm (1999) was used to analyze geosmin and MIB from the fillets and was modified as follows. Each fillet was placed into a glass distillation flask. The flask was then heated in a microwave oven (Daewoo, model TMW-1100EC) for 4 min 45 s at power level '4' while purging with 80 ml l^{-1} of N_2 gas. The collected distillate was cooled in a polystyrene box filled with ice, and the volume was adjusted to 25 ml using Nanopure water. Each 25 ml sample was placed into a 40 ml glass vial containing 6 g of NaCl. Each vial was sealed with a crimp cap. The vials were stored at 4°C until the SPME-GC-MS analysis. Water geosmin content was determined in the same manner. Standard solutions (Supelco) were used to determine the molecular ion base peaks and the retention time. These values were monitored at specific mass to charge ratios (*m/z*) 95, 135 and 168 for 2-MIB and at *m/z* 112, 126 and 182 for geosmin. The retention time was 11.7 min for MIB and 19.7 min for geosmin. The limit of quantification was 0.001 µg kg^{-1} in fish flesh, whereas the limit of detection by human senses is approximately 0.6 µg kg^{-1} (Cook et al. 2001, Schrader & Rimando 2003). The human sensory threshold for geosmin in rainbow trout has been placed at 0.1 µg kg^{-1} fish (Petersen et al. 2011). The protocol for detecting geosmin and 2-MIB using GC-MS was previously described by Lloyd & Grimm (1999) and Auffret et al. (2011).

Water analysis

Determination of the concentration of inorganic anions and cations was performed on duplicate samples that were derived from the effluent of the fish culture tanks. Water samples (30 ml) were filtered through a 0.45 µm filter, and filtrates were analyzed (50 µl volume injection) using ion chromatography (ICS-3000, Dionex). Cations were analyzed with an IonPac AS17 column (4 × 250 mm) with a KOH eluent gradient (9–30 mM, for 16 min) with 1 ml min^{-1} flow rate at 30°C. Anions were analyzed with an IonPac CS16 column (5 × 250 mm) with methanesulfonic acid (42 mM) as eluent with 1 ml min^{-1} flow rate for 16 min at 30°C. Analyses were performed according to methods described in the Appendix. The nitrite samples were analyzed according to the colorimetric method using a HACH DR-2000 spectrophotometer (HACH method 8155: salicylate, and method 8507: diazotization, respectively). Transition metal concentrations were determined using an anion exchange

chromatography technique (ICS-3000, Dionex). The same analyses were performed according to the method described by Auffret et al. (2013) and furnished in the Appendix.

Statistical analysis

All data comparing LP and HP diets were subjected to the 2-tailed *t*-test with equal variance. Statistical analyses were carried out using the IBM SPSS program for Windows (v. 20.0). The significance of any apparent differences between mean values was determined at the 95% level of confidence ($p < 0.05$), unless otherwise stated. Pearson correlation coefficients and the significance of any differences were analyzed for tank water P, water geosmin and fish fillet geosmin data using SPSS.

RESULTS

Trout reared on the LP diet had very slightly but not significantly greater ($p > 0.05$) final mean biomass gain than those fed the HP diet (Table 2). FCR varied between 1.08 and 1.24. The FCRs for LP replicates were comparable to that of the HP diet. Neither TGC (0.12) nor SGR (0.91–0.94) was significantly influenced by the dietary treatments throughout the experimental period.

The digestibility of dry matter, protein, lipid and ash varied significantly between the 2 diets (Table 3), and were significantly higher ($p < 0.05$) in fish fed the LP diet (73.9 ± 0.4%, 91.6 ± 1.0%, 84.4 ± 0.7%, and 44.6 ± 0.85%, respectively) compared to fish fed the HP diet (70.4 ± 1.2%, 85.1 ± 0.4%, 78.1 ± 0.9% and 37.3 ± 2.9% respectively). The digestibility of energy was not significantly influenced by the diets.

The ADC of P was significantly higher ($p = 0.034$) in fish fed the LP diet compared to fish fed the HP diet. The values for P retention at the end of the experiment were not significantly different, though P intake varied widely in the 2 diets (Fig. 1A). Neither nitrogen (except solid) nor energy fractions were influenced by the dietary treatments (Fig. 1B,C). Regarding the concentrations of dissolved and solid P waste, there was a pronounced effect of the dietary P concentrations. Both the dissolved and solid P waste output were significantly higher from fish fed the HP diet (3.0 ± 0.8 and 6.0 ± 0.2 g kg^{-1} fish, respectively) than fish fed the LP diet (0.2 ± 0.6 and 2.3 ± 0.1 g kg^{-1} fish, respectively) (Fig. 1A). Similarly, trout fed the HP diet showed significantly ($p < 0.001$) higher concentrations of phosphate in tank water for every sampling event until the end of the experiment (Table 4) than fish fed the LP diet. The concentrations of inorganic anions (NO_2^-, Cl^-, SO_4^{2-}) were not significantly different in the tank water of the fish fed the different diets on some sampling events. The cations, e.g. Fe^{3+} and Cu^{2+}, were not significantly different in the tank water of the fish fed the different diets in all the sampling events throughout the experimental period (Table 5). The cations, e.g. Na^+ and Ca^{2+}, showed no significant difference in the tank water of the fish fed the different diets on some sampling events. Magnesium (Mg^{2+}), potassium (K^+) and zinc (Zn^{2+}) concentrations in tank water were significantly lower for fish

Table 2. Mean individual weight (wt) gain, feed conversion ratio (FCR) (see Eq. 2 in 'Materials and methods'), specific growth rate (SGR) (see Eq. 3) and thermal-unit growth coefficient (TGC) (see Eq. 4) for rainbow trout *Oncorhynchus mykiss* fed low-phosphorus (LP) and high-phosphorus (HP) diet. Survival rate = (Final number of fish/Initial number of fish) × 100. Values are mean ± SD (n = 4 tanks per diet). na: not applicable

Parameter	Day	Treatment		p
		LP	HP	
Individual wt (g)	0–21	159.5 ± 6.7	159.1 ± 4.0	0.496
	21–42	190.0 ± 6.3	189.9 ± 2.9	0.618
	42–63	228.1 ± 10.0	229.0 ± 5.4	0.604
	63–84	275.6 ± 8.9	277.3 ± 2.4	0.311
Final mean wt gain (g)		146.8 ± 0.0	151.4 ± 5.1	0.380
FCR	0–21	1.17 ± 0.03	1.08 ± 0.18	0.464
	21–42	1.09 ± 0.29	1.11 ± 0.11	0.897
	42–63	1.15 ± 0.28	1.21 ± 0.08	0.748
	63–84	1.17 ± 0.00	1.24 ± 0.00	0.682
Final mean FCR		1.14 ± 0.13	1.15 ± 0.06	0.480
SGR	0–21	1.02 ± 0.04	1.10 ± 0.15	0.360
	21–42	0.78 ± 0.20	0.74 ± 0.07	0.688
	42–63	0.93 ± 0.80	0.84 ± 0.06	0.535
	63–84	0.82 ± 0.00	0.89 ± 0.00	0.440
Final mean SGR		0.94 ± 0.00	0.91 ± 0.02	0.180
TGC	0–21	0.11 ± 0.00	0.13 ± 0.01	0.383
	21–42	0.09 ± 0.02	0.09 ± 0.01	0.667
	42–63	0.12 ± 0.01	0.11 ± 0.00	0.558
	63–84	0.11 ± 0.00	0.16 ± 0.00	0.417
Final mean TGC		0.12 ± 0.00	0.12 ± 0.00	0.170
Total feed fed (kg)		5.40 ± 0.00	5.40 ± 0.00	na
Survival rate (%)		99.75 ± 0.50	100.00 ± 0.00	0.390

Table 3. Apparent digestibility coefficient (ADC) (see Eq. 8 in 'Materials and methods') for dry matter, phosphorus, protein, lipid, energy and ash of rainbow trout *Oncorhynchus mykiss* fed low-phosphorus (LP) and high-phosphorus (HP) diet. Values are mean ± SD (n = 4 tanks per diet). Significant values ($p < 0.05$) in **bold**

	Diet		p
	LP	HP	
Dry matter	73.91 ± 0.40	70.40 ± 1.16	**<0.001**
Phosphorus	59.30 ± 1.09	53.17 ± 2.79	**0.034**
Protein	91.57 ± 0.96	85.13 ± 0.43	**<0.001**
Lipid	84.36 ± 0.67	78.12 ± 0.90	**0.012**
Energy	75.97 ± 0.48	73.47 ± 0.30	0.760
Ash	44.60 ± 0.85	37.32 ± 2.92	**0.018**

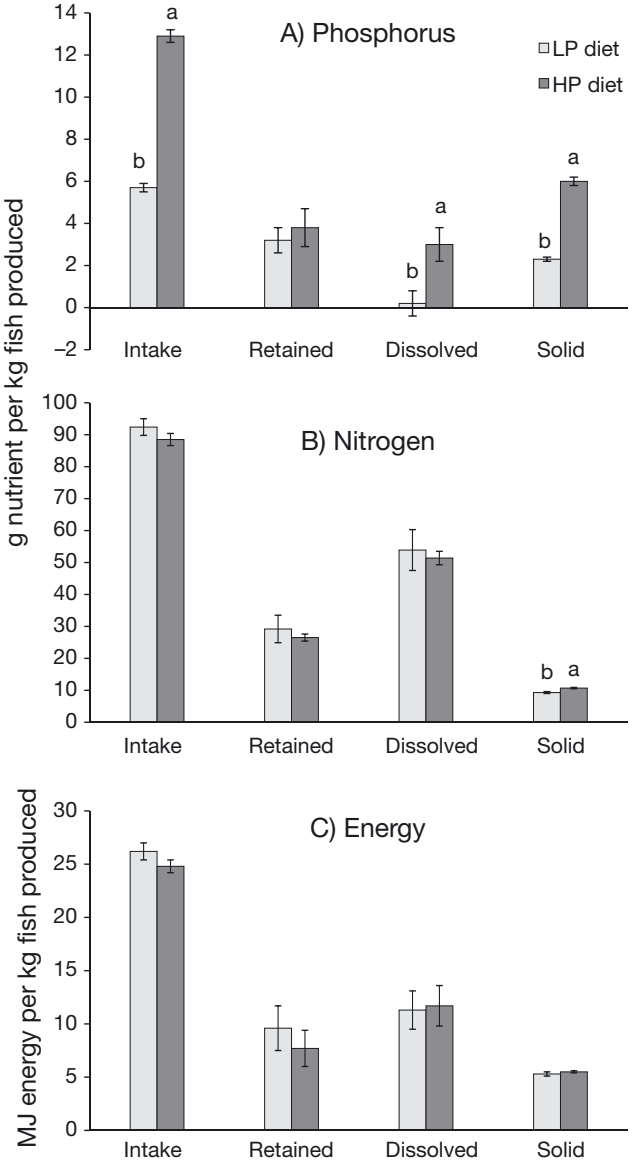

Table 4. Concentration (ppm) of inorganic anions in tank water at each sampling event for rainbow trout *Oncorhynchus mykiss* fed low-phosphorus (LP) and high-phosphorus (HP) diet. Values are mean ± SD (n = 4 tanks per diet). Significant values ($p < 0.05$) in **bold**

Parameter	Day	Treatment		p
		LP	HP	
Phosphate (PO_4^{3-})				
	0	0.58 ± 0.10	0.51 ± 0.01	**<0.010**
	21	0.00 ± 0.00	3.87 ± 0.02	**<0.010**
	42	0.00 ± 0.00	6.45 ± 0.10	**<0.010**
	63	0.02 ± 0.00	6.79 ± 0.18	**<0.010**
	84	0.04 ± 0.00	7.79 ± 0.06	**<0.010**
Nitrite (NO_2^-)				
	0	0.05 ± 0.00	0.03 ± 0.01	0.136
	21	0.20 ± 0.00	0.12 ± 0.00	**<0.010**
	42	0.16 ± 0.00	0.17 ± 0.00	**0.046**
	63	0.17 ± 0.00	0.17 ± 0.00	0.201
	84	0.16 ± 0.00	0.17 ± 0.00	**0.018**
Nitrate (NO_3^-)				
	0	49.06 ± 0.28	34.69 ± 0.18	**<0.010**
	21	101.28 ± 3.64	69.94 ± 1.84	**<0.010**
	42	148.70 ± 4.15	100.04 ± 1.91	**<0.010**
	63	125.70 ± 10.64	107.17 ± 3.29	**0.036**
	84	177.30 ± 6.55	124.59 ± 0.79	**<0.010**
Chloride (Cl^-)				
	0	44.99 ± 0.18	39.48 ± 0.19	**<0.010**
	21	39.39 ± 1.44	39.86 ± 0.57	0.517
	42	42.33 ± 1.13	37.59 ± 0.72	**0.014**
	63	26.19 ± 2.22	28.70 ± 2.78	0.385
	84	24.64 ± 0.93	23.22 ± 0.13	**0.040**
Sulfate (SO_4^{2-})				
	0	19.93 ± 0.41	18.51 ± 0.40	**0.011**
	21	22.69 ± 0.87	21.11 ± 0.84	**0.043**
	42	29.00 ± 1.05	24.60 ± 0.48	**0.010**
	63	23.22 ± 2.01	23.38 ± 0.76	0.880
	84	31.46 ± 0.90	26.08 ± 0.23	**<0.010**

fed the HP diet than the LP diet for most of the sampling events (Table 5).

The geosmin concentrations of trout fillets were higher in fish fed the HP diet versus the LP diet. A pairwise comparison showed significantly higher geosmin levels in fish fillet with fish fed the HP diet (3.13 µg kg^{-1} and 2.42 µg kg^{-1}) than fish fed the LP diet (1.29 µg kg^{-1} and 1.36 µg kg^{-1}) for Weeks 3

Fig. 1. *Oncorhynchus mykiss*. (A) Phosphorus, (B) nitrogen and (C) energy intake, retention and loading estimated at the end of the feeding trial from larger rainbow trout fed low-phosphorus (LP) and high-phosphorus (HP) diet. Mean values (n = 4 tanks per diet) in a column with different superscripts are significantly different ($p < 0.05$). For calculations of nutrient retention, see Eq. (5) in 'Materials and methods'; solid nutrient load, see Eq. (6); and dissolved nutrient load, see Eq. (7)

Table 5. Concentration (ppm) of inorganic cations (magnesium, sodium, calcium, potassium) and metals (iron, copper, zinc) in tank water at each sampling event for rainbow trout *Oncorhynchus mykiss* fed low-phosphorus (LP) and high-phosphorus (HP) diet. Values are mean ± SD (n = 4 tanks per diet). Significant values ($p < 0.05$) in **bold**

Parameter	Day	Treatment		p
		LP	HP	
Magnesium (Mg^{2+})				
	0	2.60 ± 0.03	2.48 ± 0.03	**0.040**
	21	3.22 ± 0.02	2.95 ± 0.00	**<0.010**
	42	2.42 ± 0.09	2.42 ± 0.03	0.972
	63	2.18 ± 0.03	2.09 ± 0.02	**0.046**
	84	2.31 ± 0.04	2.14 ± 0.03	**0.020**
Sodium (Na^{+})				
	0	52.00 ± 0.84	45.46 ± 1.17	**0.004**
	21	70.12 ± 4.90	52.58 ± 17.76	0.194
	42	104.25 ± 3.60	92.39 ± 1.90	**0.017**
	63	75.54 ± 5.86	80.84 ± 2.55	0.140
	84	95.88 ± 5.31	77.29 ± 0.72	**<0.010**
Calcium (Ca^{2+})				
	0	24.32 ± 0.28	22.27 ± 0.39	**0.006**
	21	17.04 ± 0.08	16.67 ± 0.16	**0.038**
	42	14.24 ± 0.24	14.81 ± 0.17	0.072
	63	12.65 ± 0.21	13.60 ± 0.13	**<0.010**
	84	11.77 ± 0.27	13.61 ± 0.16	**<0.010**
Potassium (K^{+})				
	0	1.97 ± 0.01	1.76 ± 0.01	**0.024**
	21	2.05 ± 0.00	1.67 ± 0.02	**<0.010**
	42	2.32 ± 0.00	1.81 ± 0.10	**<0.010**
	63	2.24 ± 0.00	1.93 ± 0.18	**<0.010**
	84	3.09 ± 0.00	2.53 ± 0.06	**<0.010**
Iron (Fe^{3+})				
	0	0.62 ± 0.78	0.02 ± 0.04	0.221
	21	2.95 ± 0.77	2.95 ± 0.82	0.180
	42	2.35 ± 0.74	2.35 ± 0.00	0.320
	63	2.42 ± 0.34	2.42 ± 2.03	0.393
	84	2.78 ± 0.70	2.78 ± 1.41	0.090
Copper (Cu^{2+})				
	0	1.03 ± 0.44	1.31 ± 1.04	0.639
	21	1.46 ± 0.42	1.35 ± 0.19	0.473
	42	1.49 ± 0.48	0.94 ± 0.42	0.434
	63	1.05 ± 0.21	1.05 ± 0.42	0.594
	84	1.35 ± 0.54	0.62 ± 0.33	0.117
Zinc (Zn^{2+})				
	0	5.38 ± 0.29	4.37 ± 1.26	0.402
	21	6.83 ± 1.35	4.44 ± 0.50	0.051
	42	6.35 ± 0.52	3.65 ± 0.58	**0.001**
	63	6.38 ± 0.59	4.73 ± 0.74	**0.017**
	84	10.77 ± 2.30	7.02 ± 0.44	**0.048**

(Day 21) and 6 (Day 42), respectively (Fig. 2). However, there was no significant difference detected between the 2 diets at the Week 8 (Day 56) and Week 12 (Day 84) sampling events, although considerable variability in concentrations was observed. Geosmin concentrations of the tank water, biofilter

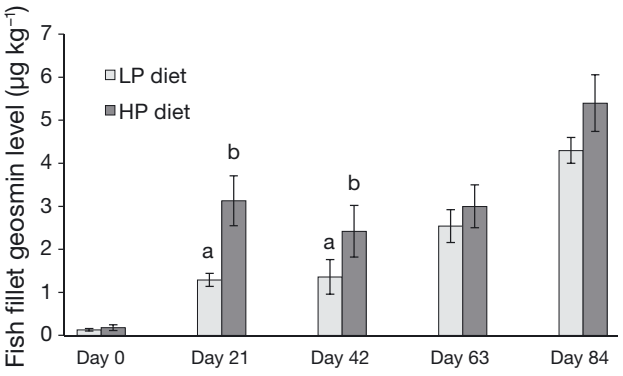

Fig. 2. *Oncorhynchus mykiss*. Concentration of geosmin in rainbow trout flesh fed low-phosphorus (LP) and high-phosphorus (HP) diet for 84 d. Data are means (±SD) of 4 tanks (3 fish per tank) per diet. Different letters above bars indicate significant difference at $p < 0.05$

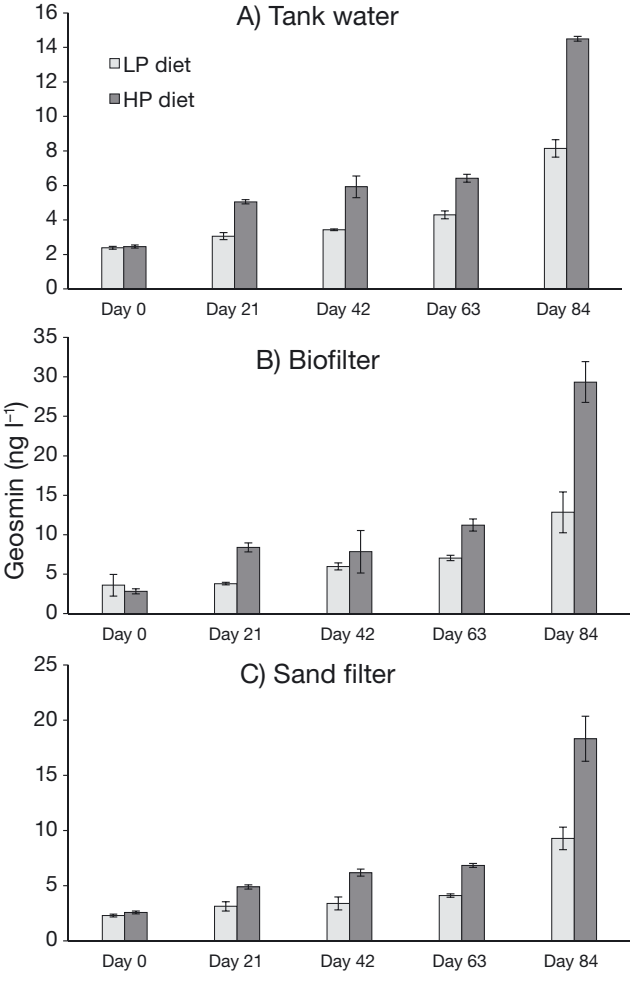

Fig. 3. *Oncorhynchus mykiss*. Changes in geosmin concentration in the (A) tank water, (B) biofilter and (C) sand filter of the tanks where fish received low-phosphorus (LP) and high-phosphorus (HP) diet for 84 d. Data are means (±SD) of 4 tanks per diet

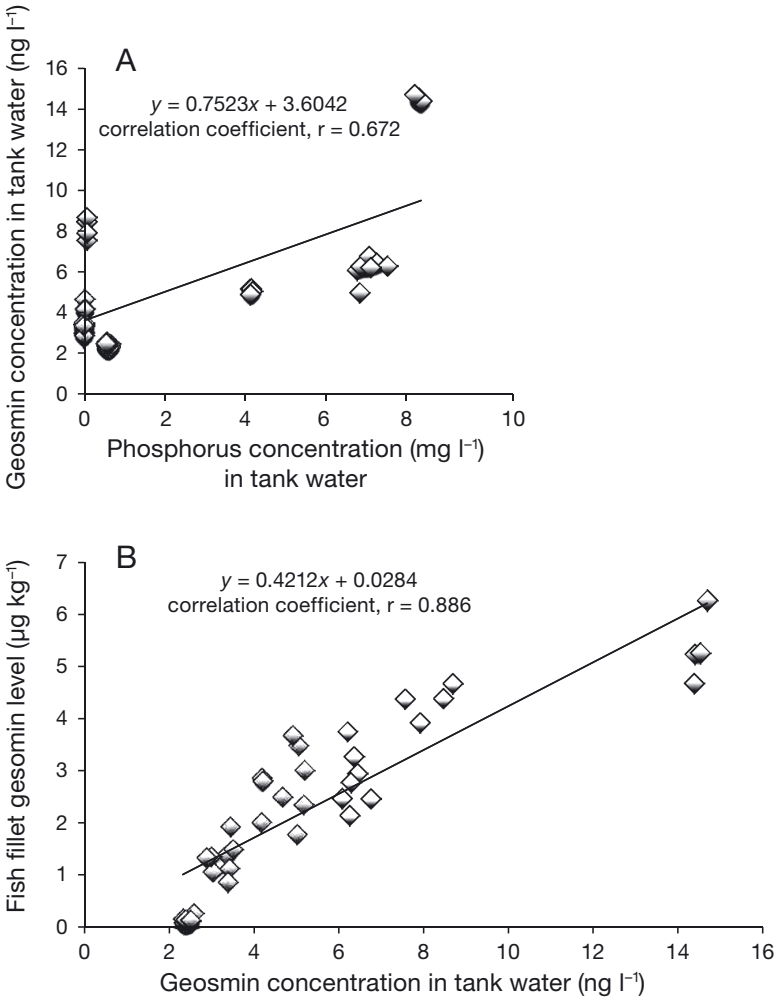

Fig. 4. *Oncorhynchus mykiss*. Linear correlations between (A) the phosphorus and geosmin concentrations in tank water and (B) the geosmin concentration in tank water and that in fish fillets from the feeding trial where fish received low-phosphorus (LP) and high-phosphorus (HP) diet. Data are means (±SD) of 4 tanks per diet. Correlations are significant at $p < 0.0001$

and sand filter were higher for the fish that received the HP diet versus the LP diet in all sampling events; however, the difference was not significant between the 2 diets (Fig. 3A–C). Although there was no strong linear relationship between soluble P concentration and geosmin in tank water when we integrated the data from both the LP and the HP diets ($y = 0.7523x + 3.6042$; $r = 0.672$; $p < 0.0001$) (Fig. 4A), the relationship was stronger in the HP diet (data not shown). Furthermore, there was a strong proportional linear relationship between the geosmin concentration in tank water and geosmin concentration in fish fillet of the fish fed the LP diet and HP diet ($y = 0.4212x + 0.0284$; $r = 0.886$; $p < 0.0001$) (Fig. 4B).

DISCUSSION

The reduction of P waste output from aquaculture operations is considered a key element for the long-term sustainability of aquaculture. The most direct method of reducing P loading is achieved by manipulation of the P concentration in feed and improvement of diet digestibility. Current commercial trout feeds usually have a total P content that varies from 10 to 14.3 g kg^{-1} diet (Sarker et al. 2011). As expected, the total P content of the HP diet (13 g kg^{-1} diet) in this study resembles the P content of commercial trout diets. The TGC and FCR values did not vary between the 2 diets; that the FCR values were identical throughout the study indicates that the protein and energy content of the diets were balanced (Cho & Kaushik 1990). In the present study, growth was not affected by the LP diet when compared to the HP diet. The growth performance of fish in the current study confirmed that the LP (5.4 g kg^{-1} diet) diet had sufficient total P to satisfy the minimum requirement for large rainbow trout. In large fish, the focus of the present study, the requirement for P is known to decrease with age because the growth rate decreases and dietary P is used mainly for maintaining metabolic functions (Lellis et al. 2004, Koko et al. 2010). The P content of the LP diet was in the range of the dietary P requirement that has been reported for normal growth of rainbow trout, 4.0–6.0 g kg^{-1} (Ketola & Richmond 1994, Rodehutscord 1996). Similar findings suggest that available P levels can be reduced in rainbow trout diets to 4.7 g kg^{-1} diet at 314 g live mass without loss in production (Rodehutscord 1996).

Fish fed the LP diet were characterized by very low dissolved and solid waste P output, suggesting that this diet approached maximum retention and the P requirement was met. Non-fecal P (urinary or gill) concentration has been shown to be a rapid and sensitive indicator of dietary P intake (Bureau & Cho 1999, Sugiura et al. 2000). Research has shown that non-fecal P (urinary or gill excretion) is significantly lower in fish fed an LP diet compared to an HP diet (Sugiura et al. 2000). In the present study, the amount of retained P by fish in the LP and HP dietary groups were not significantly different (Fig. 1A), which reflects that the physiological P needs of both dietary groups were met.

It is well known that vertebral deformities may appear long before any growth reduction is exhibited.

In juvenile Atlantic salmon *Salmo salar* and rainbow trout, it has been observed that continual feeding on a P-deficient diet eventually leads to poor bone mineralization and deformities (Vielma & Lall 1998, Fontagnè et al. 2009, Fjelldal et al. 2012). This phenomenon has also been observed in large fish. For instance, Koko et al. (2010) found that changes in weight of 164 g trout fed deficient P (4.0 g kg^{-1} diet) and sufficient P (8.0 g kg^{-1} diet) over time remained similar during the initial 56 d. After this period, poor growth performance and a number of visual deficiency signs were observed, such as bone deformities in the tail regions of trout fed the P-deficient diet. However, there were no visual signs of P deficiency in the current study. This indicates that fish fed the LP diet received an adequate level of P for bone mineralization for the 84 d feeding period. However, more work is required on the effect of an LP diet on rainbow trout health and bone mineralization in a longer-term feeding trial, which our research team is currently investigating.

Even though the diets were both isocaloric and isonitrogenous, there was a significant depression in the apparent digestibility of dry matter, P, ash, protein and lipid of fish fed the HP diet (containing 6.9 g digestible P kg^{-1} diet). There was also a substantial increase in the P waste output fractions, which could suggest that these fish were somehow negatively affected by the high P intake. Based on the results of P budgets, fish in all dietary treatment groups displayed similar fractions of retained P; however, fish in the HP dietary treatment groups excreted a comparatively significant higher amount (6 g P kg^{-1} fish produced) of solid P. Non-fecal dissolved P excretion occurs only when the diet contains available/digestible P that exceeds the requirement level (Bureau & Cho 1999, Rodehutscord et al. 2000, Sugiura et al. 2000, Sarker et al. 2009). In the present study, the dissolved P waste output was very minimal (0.2 g P kg^{-1} fish produced) from fish fed the LP diet (3.2 g digestible P kg^{-1}), increasing (3 g P kg^{-1} fish produced) significantly in the fish fed the HP diet (6.9 g digestible P kg^{-1} diet), suggesting that the dietary P requirement had been exceeded in this group. The fact that the fish fed the LP diet displayed significantly lower or borderline (negative value) dissolved P waste output presumably reflects that the fish were P-limited at the start of the trial (0 mg P l^{-1} in tank water for Days 21 and 42), therefore retaining a larger share of dietary P (Sugiura et al. 2000).

The current study seeks to clarify the impact of dietary P concentration and the magnitude of the waste P production on the appearance of off-flavor compound deposition in fish flesh and water. The P budgets balanced very well, verifying that the fate of dietary P had been satisfyingly accounted for. Fish fed the HP diet displayed higher geosmin concentrations in fillets throughout the experimental period, and the difference with the LP group was significant until Day 42. In the LP group, the fish fillet geosmin level was very low at Day 0 compared to Day 84, whereas the phosphate level in water was quite high at Day 0 (0.58 ppm) compared to Day 84 (0.04 ppm). This could be attributed to the fact that the fish were P-limited at the start of the trial, and fish receiving the LP diet displayed a net disappearance of soluble P concentration (0 mg l^{-1}) from the tank water (Table 4). They may have retained maximum P until Day 42 for their physiological requirement or could even have taken up soluble P from water via gills (Sugiura et al. 2003, Sarker et al. 2011). This disappearance of soluble P in the water indicated that P might not be stimulating the growth or proliferation of microorganisms, and coincided with the lower concentration of the geosmin level for the first 42 d in fish fed the LP diet (Findlay et al. 2009, Auffret et al. 2011). However, it should be noted that the concentration of geosmin levels in fish was high, >2.4 µg kg^{-1} fish for both diets. The human sensory threshold for geosmin in rainbow trout ranges from 0.1 to 0.9 µg kg^{-1} fish (Robertson et al. 2005, Peterson et al. 2011). This suggests that even though fish fed the LP diet contributed less geosmin concentration in fillet, further reduction is needed to achieve a level below the sensory threshold. To provide adequate conditions for depuration of off-flavors, either the fish could be moved to clean odor-free water or the geosmin could be removed from the culture water *in situ* (Robertson et al. 2005, Burr et al. 2012); e.g. rainbow trout depuration periods of ~1 wk are typically practiced (Petersen et al. 2011, 2014).

This study demonstrated that the concentration of geosmin in the rainbow trout flesh is highly correlated with the accumulated geosmin concentration in the tank water. The accumulation of tainting compounds in flesh is extremely rapid (Robertson et al. 2005) and can be intensified by the lipid content of fish (Johnsen & Lloyd 1992, Jones et al. 2013). The higher geosmin levels in the fish on Days 63 and 84 relative to Day 42 might be linked with the body lipid content of fish. Although we did not determine the relationship between lipid content of fish flesh and uptake of geosmin concentration in this study, our previous study confirmed the fact that the concentration of lipid in trout has an influence on the uptake of geosmin in water (authors' unpubl.). This suggests that due to the relationship

between geosmin uptake and lipid content, the lean fish could contribute potentially the lower concentration of taint compounds in farmed fish (Johnsen & Lloyd 1992, Robertson et al. 2005).

The aquafeeds industry has long recognized and driven research to find aquafeeds that use alternative protein ingredients, particularly plant-derived proteins (Gatlin et al. 2007). Plant-derived proteins contain high levels of phytate-bound P (Mainstone & Parr 2002, Sarker et al. 2006, 2013), which is largely (70%) indigestible to monogastric animals like fish, who lack endogenous phytase activity to liberate phytate P (NRC 1993). Thus, P ends up in fish culture effluents, which contribute to eutrophication (Mainstone & Parr 2002, Sarker et al. 2009). P balance calculations indicated that both solid and dissolved P production was higher in the HP diet than LP diets, therefore confirming that a difference in digestibility between diets was due to the difference in supplemented P concentration in the diet, not the level of phytate P, which was the same in both diets. Use of phytase enzymes to increase utilization of phytate P in the feed may be beneficial in reducing the growth of microorganisms and thus reducing the occurrence of off-flavor. However, this experiment was not designed to study the dietary P source (such as phytate) in relationship to off-flavor. Moreover, the influence of microbial activity on the phosphate concentration in water was not directly evaluated in this study. Thus, more research is certainly needed to explore the effects/consequences of phytate P with respect to P waste outputs and geosmin-associated off-flavor compounds in fish raised in RAS.

Regardless of this mechanism, it is important to note that low concentrations of P in RAS may contribute to lowering geosmin-associated off-flavor in the culture system and in fillet from fish raised in RAS. The large increase of geosmin concentration reflects the P waste output in the system water that facilitated the proliferation of microorganisms (Auffret et al. 2011). The effectiveness of P utilization by microorganisms is stimulated by the fraction of the P loading from fish culture operation that is biologically available (Findlay et al. 2009). This is due to the fact that most of the dissolved P waste excreted by the fish is in the form of orthophosphate (Bureau & Cho 1999), a component that is readily available to the microorganisms. Likewise, Redfield (1958) and Armstrong (1999) reported that available P in water is one of the key factors implicated in cyanobacterial proliferation and contributes to producing geosmin-associated off-flavor compounds in the aquaculture system (Ridal et al. 1999, Sugiura & Nakano 2000, Dzialowski et al. 2009). It has been reported that the P released from sediment induces off-flavor problems in catfish and carp ponds when the P content in the water was in the range of 0.33–0.71 mg l^{-1} (Zimba et al. 2003, Vallod et al. 2007). An amount of 1 mg l^{-1} was previously correlated with the occurrence of geosmin production in aquaculture systems (Robertson et al. 2006). This amount has also been shown to control the biosynthesis of secondary metabolites such as geosmin in *Streptomyces* sp. (Schrader & Blevins 2001, Martín 2004). A number of microorganisms can produce volatile organic compounds that affect the taste and smell of fish and water; e.g. several taxa of cyanobacteria (blue-green algae), filamentous *Actinobacteria* (actinomycetes) and *Myxobacteria* frequently produce 2 key off-flavor compounds, geosmin and 2-MIB, in aquaculture and other aquatic environments (Tucker & Martin 1991, Jüttner & Watson 2007, Auffret et al. 2011). However, in indoor RAS tank conditions, cyanobacteria might not grow as favorably as in open water ponds due to the presence of sunlight outdoors. In indoor trout tanks, the growth of non-photosynthetic microbes could be a major source of geosmin. In this study, changes in geosmin concentration in tank water, biofilter and sand filter increased over time until the end of the experiment; at every sampling event, fish receiving the HP diet showed numerically (not statistically) higher concentrations of geosmin. In this study, Pearson correlation analysis was performed and showed that the soluble P concentration in tank water was not strongly correlated (correlation coefficient, $r = 0.672$; $p < 0.0001$) with the geosmin concentration when we integrated all the data from the 2 dietary treatments; however, the relationship is even stronger (correlation coefficient, $r = 0.769$; $p < 0.0001$) when considering only fish receiving the HP diet (data not shown).

It is important to note that RAS biofilms can be a major source of the off-flavor compounds. It has been reported that in RAS, the biofilter and the sand filter are the 2 compartments that are known to concentrate geosmin producers and to accumulate phosphate (Shnel et al. 2002, Guttman & van Rijn 2008). In our previous study, we reported that the detected *Streptomyces*, *Myxococcales*, *Sorangium* and *Nannocystis* coincided with the detection of geosmin in trout raised in RAS (Auffret et al. 2011, 2013), and there was a positive correlation between *Nannocystis* in the fish tank and the detection of geosmin in fish tissue. Auffret et al. (2013) also speculated that P could be associated with the abundance of *Sorangium* and the geosmin-synthesis gene that could enhance the geosmin production in RAS.

In all sampling events of the present study, 2-MIB was recorded below detectable levels, suggesting that it had a negligible influence on earthy taint episodes in rainbow trout flesh and the water in which they were raised. To our knowledge, there are no published studies addressing the influence of dietary P on the development of off-flavor (geosmin) in trout fillet and system water. The results from this study suggest that care should be taken in the formulation of diets with high P levels that exceed the requirement levels for large fish, without considering metabolic waste outputs into the environment and the obvious risk of producing off-flavor compounds in water and fish flesh.

Several micronutrients, e.g. inorganic cations (Na^+, Ca^{2+}, Fe^{3+}, Cu^{2+}) in the tank water of fish fed both diets tested in this study were not significantly different. These micronutrients have not been identified as having considerable influence on the yield of several bacterial metabolites like geosmin. It has been previously reported that Ca^{2+}, K^+ and Cu^{2+} usually do not affect the secondary metabolism of bacteria and geosmin production (Schrader & Blevins 2001). However, significantly lower concentration of zinc in the tank water of fish fed the HP diet could stimulate the secondary metabolism of bacteria and permit greater geosmin production, as has been reported previously (Weinberg 1989). In the case of inorganic anions, with the exception of PO_4^{3-} concentration (significantly higher), some other anions such as (NO_2^-, Cl^-, SO_4^{2-}) were not found significantly different in the tank water of fish fed both the HP and LP diets on some sampling events. The impact of differing levels of these micronutrients in water is not well understood. Therefore, in the current study, it is unclear how metabolic P waste in water could interact with other environmental parameters and regulate the synthesis of geosmin in water and fish tissue, so further research is needed to elucidate this.

CONCLUSION AND IMPLICATIONS

The results of this study suggest that off-flavor compounds in water and fish fillets raised in RAS were related to the P concentration in the diet and consequent metabolic P waste output into the system. This study provides evidence that nutritional strategies and utilization of low-P feed in larger rainbow trout lead to significant reduction of P waste outputs and an off-flavor compound (geosmin) in RAS systems and consequentially in fillets without compromising fish growth and productivity.

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Appendix. Ion chromatography methods. Inorganic anions included chloride, nitrate, nitrite, phosphate and sulfate; inorganic cations included calcium, magnesium, potassium and sodium; metals included iron (III) and the divalent cations of copper and zinc

	Conditions
Anions	
Volume injected	50 µl
Analytical column	IonPac AS17 (4 × 250 mm)
Guard column	AG17 (4 × 50 mm)
Eluent gradient	Potassium hydroxide (9 to 30 mM), 16 min
Standard flow rate	1 ml min^{-1}
Temperature	30°C
Suppressor	Anion self-regenerating suppressor (ASRS ULTRA II, –4 mm)
Cations	
Volume injected	50 µl
Analytical column	IonPac CS16 (5 × 250 mm)
Guard column	CG16 (5 × 50 mm)
Eluent	Methanesulfonic acid (MSA) (42 mM), 16 min
Standard flow rate	1 ml min^{-1}
Temperature	30°C
Suppressor	Cation self-regenerating suppressor (CSRS ULTRA II, –4 mm)
Metals	
Volume injected	50 µl
Analytical column	IonPac CS5A (4 × 250 mm)
Guard column	CG5A (4 × 50 mm)
Eluent	MetPac PDCA eluent (5×)
Standard flow rate	1.2 ml min^{-1}
Post-column reagent	PAR (0.5 mM)
Reagent flow rate	0.7 ml min^{-1}
Detector wavelength	520 nm

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