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The impact of elevated water ammonia and nitrate concentrations on physiology, growth and feed intake of pikeperch (*Sander lucioperca*)

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ABSTRACT

The ammonia (NH_3) and nitrate (NO_3^-) threshold concentrations in rearing water of juvenile pikeperch (*Sander lucioperca*) were assessed. Pikeperch with an initial mean (SD) weight of 17.7 (4.2) g were exposed to 0.9 (control), 3.6, 5.2, 7.1, 11.2 and 18.9 μM NH_3 in the water for 42 days. Plasma NH_4^+ concentrations stayed at control levels (~650 μM) up to 11.2 μM NH_3 in the water. At the highest water NH_3 concentration tested, plasma NH_4^+ had more than doubled to 1400 μM. Based on the specific growth rate, the EC_{10} value for NH_3 was 5.7 μM. When pikeperch (initial mean (SD) weight of 27.0 (4.9) g) were exposed to 0.1 (control), 1.5, 2.3, 3.7, 6.1, 10.2, 15.8 and 25.6 mM NO_3^- for 42 days, mean (SD) plasma NO_3^- concentrations increased linearly from 88 (47) to 5993 (899) μM at the highest ambient NO_3^- level. Feed intake, specific growth rate and feed conversion ratio were not affected. Neither NH_3 nor NO_3^- exposure significantly affected haematocrit, plasma concentrations of cortisol, glucose, lactate, osmolality, gill morphology or branchial Na^+/K^+-ATPase activity in pikeperch. For juvenile pikeperch we advise not to exceed a water NH_3 concentration of 3.4 μM (0.05 mg NH_3–N/L), the lower limit of the 95% confidence interval of the EC_{10} value for SGR, to ensure proper physiology and growth. For NO_3^- we advise not to exceed 25 mM (350 mg NO_3^-–N/L). This criterion is based on the highest NO_3^- concentration tested (25.6 mM). As no negative effects were detected at the highest concentration tested, the actual NO_3^- threshold probably exceeds 25.6 mM.

1. Introduction

Aquaculture of pikeperch (*Sander lucioperca*) is important for the diversification of freshwater aquaculture production in Northern and Central Europe, and an attractive alternative for common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*). Among aquaculturists interest increases to intensify aquaculture of pikeperch (Müller-Belecke and Zienert, 2008). In intensive recirculating aquaculture systems (RAS) fish run the risk of significant exposure to nitrogenous wastes including ammonia (NH_3), nitrite (NO_2^-) and nitrate (NO_3^-). Nitrogenous waste is produced by fish through protein catabolism (Wood, 1993). The majority of teleostean fishes, including pikeperch, are ammonotelic and excrete most of their nitrogenous waste as ammonia across the gills to the water (Wilkie, 2002). High water ammonia leads to rapid accumulation of ammonia in plasma and tissues (Wright et al., 2007), where, at physiological pH, it is mainly present as NH_4^+ (Wilkie, 2002). High internal ammonia is neurotoxic (Cooper and Plum, 1987 in Wilkie, 2002). High water ammonia is an important limiting factor for intensive aquaculture (Boeuf et al., 1999) and should therefore be kept below a defined species-specific threshold.

In RAS, ammonia in the culture water is controlled by conversion of ammonia to NO_3^- in aerobic biofilters. NO_3^- subsequently accumulates in the culture water (Bovendeur et al., 1987; Eding et al., 2006) and fish farmed in RAS may be chronically exposed to NO_3^- levels up to 70 mM (1000 mg N/L; Van Rijn, 2010). High water NO_3^- results in the appearance of NO_3^- in the plasma compartment (Schram et al., in press; Stormer et al., 1996). Uptake of NO_3^- via the gills is low compared to that of ammonia and nitrite due to an apparently low branchial permeability for NO_3^- (Stormer et al., 1996). NO_3^- is less toxic than nitrite and ammonia (Scott and Crunkilton, 2000). Chronic exposure to high NO_3^- however, can lead to reduced feed intake, and growth (Schram et al., in press). In addition, nitrate was identified as being potentially associated with health problems observed in rainbow trout reared in RAS with near zero water exchange (Davidson et al., 2011).

We hypothesized that chronic exposure of juvenile pikeperch to NH_3 or NO_3^- above certain threshold concentrations result in physiological

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disturbance and reduced growth. Neither NH_3 nor NO_3^- threshold concentrations have been established for juvenile pikeperch. As a result it is unclear whether intensive farming of pikeperch at high water NH_3 or NO_3^- results in physiological disturbance and reduced growth. To test this hypothesis and to establish threshold concentrations, we exposed juvenile pikeperch to increased water NH_3 and NO_3^- levels for 42 days.

2. Materials and methods

2.1. Experimental fish

Juvenile pikeperch (*S. lucioperca*) were obtained from Excellence Fish BV, Horst, The Netherlands. Husbandry and experimentation were in accordance with the Dutch law on animal welfare, and approved by the ethical committee for animal experimentation of Wageningen UR Livestock Research (number 2012053.b for the NH_3 experiment, number 2012021.b for the NO_3^- experiment).

2.2. Experimental exposure system

During acclimatization and the experimental period of both experiments, all aquaria were supplied with local tap water at a flow rate of 500 L/d. Experimental NH_3 concentrations were realised by addition of NH_4Cl stock solutions (Table 1). Sodium bicarbonate ($NaHCO_3$) was added to the NH_4Cl stock solutions to adjust the pH (overall pH range: 7.00–8.18; Table 1). In addition, sodium chloride (NaCl) was added to the NH_4Cl stock solutions to compensate for the differences in chloride concentrations in the aquaria arising from NH_4Cl addition. Total predicted sodium concentrations in the aquaria from $NaHCO_3$ and NaCl combined were equal among treatments (Table 1). Fresh stock solutions were prepared daily during the first six days of the experimental period. During the remainder of the experimental periods fresh stock solutions were prepared twice per week. To prevent evaporation of NH_3, stock solutions were stored in closed vessels and inside the vessel the surface of the stock solution was covered by a floating plastic sheet. NH_3 concentrations were gradually increased to the designated concentrations during the first six days of the experimental period.

Experimental NO_3^- concentrations were realised by addition of $NaNO_3$ stock solutions (Table 2). Fresh stock solutions were prepared daily during the first ten days of the experimental periods. During the remainder of the experimental periods fresh stock solutions were prepared twice per week for both experiments. NO_3^- concentrations were gradually increased to the designated concentrations during the first ten days of the experimental period.

All stock solutions were prepared in tap water and pumped into the aquaria by a peristaltic pump (Watson Marlow 505 S; Rotterdam, The Netherlands) at a flow rate of 5.00 L/d per aquarium.

2.3. NH_3 experiment

Juvenile pikeperch (n = 192) with a mean (SD) weight of 17.7 (4.2) g were randomly divided over 16 30-L rectangular aquaria and acclimatized to the experimental aquaria for seven days. The experiment lasted for 42 days and consisted of eight, duplicated treatments which were randomly assigned to the aquaria. Two extra aquaria were included to collect blood and plasma of untreated fish at the start of the experiment (treatment 1, t = 0). Fish in treatments 3 to 8 were exposed to one of six different NH_3 concentrations in the water: 0.9 (control), 3.6, 5.2, 7.1, 11.2 and 18.9 μM. We aimed for a test concentration range around the NH_3 threshold for chronic effects, without reaching acutely toxic NH_3 concentrations. To avoid acute toxicity, the highest test concentration was set at approximately 50% of the 96 h LC_{50} of 36 μM for the closely related walleye (*Stizostedion vitreum*) (Arthur et al., 1987). Fish in treatment 2 (pair-fed control) were kept at control (0.5 μM) NH_3 levels and fed the same feed ration as the fish kept at the highest (18.9 μM) NH_3 level to discriminate between effects caused by low feed intake and exposure to a high NH_3 concentration in the water.

2.4. NO_3^- experiment

Juvenile pikeperch (n = 240) with an individual mean (SD) weight of 27.0 (4.9) g were randomly divided over 20 30-L rectangular aquaria and acclimatized to the experimental aquaria for 14 days. The NO_3^- experiment lasted for 42 days and consisted of ten duplicated treatments, which were assigned randomly to the aquaria. Two extra aquaria (treatment 1, t = 0) were included to collect blood and plasma of untreated fish at the start of the experiment. In treatments 3 to 10 fish were exposed to one of eight different NO_3^- concentrations in the water: 0.1 (control), 1.5, 2.3, 3.7, 6.1, 10.2, 15.8 and 25.6 mM. Fish in treatment 2 (pair fed control) were kept at control (0.1 mM) NO_3^- levels and pair-fed to the fish kept in 25.6 mM NO_3^-.

2.5. Water quality

All aquaria were equipped with an air stone to guaranty good mixing of the added stock solutions with the aquarium water. Flow rates were monitored daily and adjusted when necessary to reach the desired NH_3 or NO_3^- concentrations. Total ammonia ($T_{Amm} = NH_3 + NH_4^+$) and NO_3^- concentrations were monitored in the respective experiments (Spectroquant cell tests for total ammonia and NO_3^-–N, Merck, Darmstadt, Germany, Hach Lange DR2800 spectro-photometer, Germany). In both experiments water samples were collected twice per week from all aquaria at approximately 11 am, 1 h after the first feeding session of the day. NH_3 concentrations were calculated from the temperature, pH and salinity dependent molar fraction of NH_3 and the measured T_{Amm} concentrations, using Table 9 in http://fisheries.org/hatchery. NH_3 concentrations were calculated to account for any variation in pH among NH_3 treatments. In both experiments water temperature, pH

Table 1
Composition of the treatment specific stock solutions in the NH_3 experiment, the predicted[a] total ammonia, ammonia, sodium and chloride concentrations and salinity and the measured values per treatment for NH_3 and total ammonia (T_{Amm}) concentration, conductivity and the pH range in the aquaria.

NH_3 treatment	Composition of stock solutions			Predicted concentrations in the aquaria				Measured water quality				
	$[NH_4Cl]$	$[NaHCO_3]$	[NaCl]	$[NH_3]$[b]	$[Na^+]$	$[Cl^-]$	Total dissolved solids	$[NH_3$–N]	$[NH_3]$	$[T_{Amm}]$	Conductivity	pH range
	(g/L)	(g/L)	(g/L)	(μM)	(mM)	(mM)	(g/L)	(mg/L)	(μM)	(mM)	(μS/cm)	
2 — Pair fed control	0	0	50.5	0	8.7	5.2	0.50	0.01	0.46	0.01	1752	7.34–8.18
3 — Control	0	0	50.5	0	8.7	5.2	0.50	0.01	0.90	0.03	1668	7.60–8.05
4 — NH_3	2.36	3.0	48.5	5.7	8.7	5.2	0.53	0.05	3.55	0.18	1557	7.00–7.96
5 — NH_3	3.27	4.3	47.5	7.7	8.7	5.2	0.55	0.07	5.16	0.24	1544	7.14–7.97
6 — NH_3	4.63	6.0	46.4	11.1	8.7	5.3	0.56	0.10	7.12	0.35	1579	7.14–7.95
7 — NH_3	6.55	8.5	44.7	15.8	8.7	5.3	0.59	0.16	11.2	0.46	1531	7.18–7.96
8 — NH_3	9.18	12.0	42.3	22.1	8.7	5.4	0.63	0.26	18.9	0.73	1643	7.33–8.01

[a] Based on equal flow rates per tank of 5 L/day for the stock solutions and 500 L/day for the tap water flow.
[b] Based on a pH of 7.4 and a water temperature of 24 °C.

Table 2
Composition of the treatment specific stock solutions in the NO_3^- experiment, the predicted[a] nitrate and sodium concentrations, the predicted salinity in all treatments and the measured values per treatment for nitrate concentration, conductivity and the pH range.

NO_3^- treatment	Stock solutions	Predicted[a] water quality			Measured water quality			
	[$NaNO_3$]	[NO_3^-]	[Na^+]	Total dissolved solids	[NO_3^-–N]	[NO_3^-]	Conductivity	pH range
	(g/L)	(mM)	(mM)	(g/L)	(mg/L)	(mM)	(μS/cm)	
2 — Pair fed control	0	0	0	0	1.4	0.1	684	7.63–7.96
3 — Control	0	0	0	0	1.4	0.1	685	7.39–7.97
4 — NO_3^-	12.1	1.4	1.4	0.12	20	1.5	841	7.61–7.95
5 — NO_3^-	20.0	2.4	2.4	0.20	32	2.3	932	7.15–8.03
6 — NO_3^-	33.1	3.9	3.9	0.33	52	3.7	1101	7.10–7.97
7 — NO_3^-	54.5	6.4	6.4	0.54	85	6.1	1377	7.63–7.98
8 — NO_3^-	90.0	10.6	10.6	0.89	143	10.2	1832	7.59–7.94
9 — NO_3^-	148.5	17.5	17.5	1.47	221	15.8	2418	7.55–7.95
10 — NO_3^-	245.0	28.8	28.8	2.42	359	25.6	3504	7.64–7.93

[a] Based on equal flow rates per tank of 5 L/day for the stock solutions and 500 L/day for the tap water flow.

and dissolved oxygen concentrations (Hach Lange HQ 40 multimeter, Germany) and conductivity (WTW Cond 315i) were monitored daily in all aquaria prior to the first daily feeding session (Tables 1 and 2). Dissolved oxygen ranged 6.7 to 8.1 mg/L in the NH_3 experiment and from 6.7 to 7.0 mg/L in the NO_3^- experiment. Water temperatures were 23.8 °C in the NH_3 experiment and 23.0 °C in the NO_3^- experiment. pH ranged from 7.00 to 8.18 in the NH_3 experiment and from 7.10 to 7.98 in the NO_3^- experiment.

2.6. Blood and plasma sampling

One day before exposure to NH_3 or NO_3^- started (day 0), fish in treatment 1 (t = 0) were sampled. After 42 days of exposure, the fish from the remaining treatments were sampled (12 fish per aquarium). Fish were rapidly netted and anaesthetised in 0.1% (v/v) 2-phenoxyethanol (Sigma, St. Louis, USA). Within 2 min, blood was taken by puncture of the caudal vessels with a syringe fitted with a 25-gauge needle. Na_2EDTA (NH_3 experiment) or heparin (NO_3^- experiment) was used as anti-coagulant. A 10 μL aliquot blood was used to assess haematocrit, the remainder was immediately centrifuged for 10 min (14,000 *g*, 4 °C) and plasma was stored at −20 °C until further analyses.

2.7. Plasma NO_3^- concentration — NO_3^- experiment

NO_x (the sum of NO_2^- and NO_3^-) was measured with a commercial nitrate/nitrite colorimetric assay kit (Cayman Chemical Company, Ann Arbor, Michigan, USA). Prior to measurement, plasma samples were filtered using a Millipore Ultra-free MC filter device (0.1 μm pore size) to remove haemoglobin and reduce background absorbance and improve colour formation with Griess reagents. Samples of 80 μL (in duplicate) were diluted in the assay buffer and then incubated for 3 h at room temperature with 10 μL of Enzyme Co-factor mixture and 10 μL of NO_3^--reductase mixture. Fifty microliters of the first Griess reagent (R1) and of the second Griess reagent (R2) was added and absorbance read at 530 nm in a Wallac 1420 VICTOR^2 counter (Turku, Finland). Due to low plasma volumes and interference with heparin plasma NO_2^- could be determined in only a subset of samples. All NO_2^- values were below 35 μM, which we interpret to indicate that plasma NO_x consists primarily of NO_3^-.

2.8. Plasma NH_4^+ — NH_3 experiment

Plasma NH_4^+ was determined using a commercial kit (Instruchemie, Delfzijl, The Netherlands), with a protocol adapted for a 96-well microplate application.

2.9. Plasma concentrations of cortisol, glucose, lactate, plasma osmolality and branchial Na^+/K^+-ATPase activity

Plasma cortisol was determined by radioimmunoassay as described in detail by Metz et al. (2005). Plasma osmolality was measured using a cryoscopic osmometer (Osmomat 030, Gonotec, Germany). Plasma glucose and lactate were measured with commercial enzymatic test kits (Instruchemie, Delfzijl, The Netherlands), with protocols adapted to a 96-well microplate. For glucose, 10 μL sample or standard (5.55 mM glucose) was mixed with 200 μL reagent and incubated for 10 min at 25 °C. Absorbance was read within 60 min at 495 nm. For lactate, 10 μL sample or standard (4.44 mM lactate) or blank (8% perchloric acid) was mixed with 290 μL of lactate reagent and incubated for 20 min at 37 °C. Absorbance was read at 355 nm. Branchial Na^+/K^+-ATPase activity was measured as described by Metz et al. (2003).

2.10. Gill morphology

From each sampled fish the second gill arch was removed immediately after blood sampling and placed overnight in Bouin's fixative (75 volumes saturated picric acid, 25 volumes saturated formaldehyde, and 5 volumes acetic acid) and embedded in paraffin. Gill sections through the trailing edge of the filament, where the chloride cells reside, were immunostained according to Dang et al. (2000). After removal of the paraffin, blocking of endogenous peroxidase with 2% (v/v) H_2O_2 and blocking of non-specific sites with 10% (v/v) normal goat serum, slides were incubated overnight with a monoclonal antibody against chicken Na^+/K^+-ATPase (final dilution of 1:500; IgGα5, Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, USA). Goat anti-mouse (Nordic Immunology, Tilburg, The Netherlands) was used as a second antibody (1:150). The slides were subsequently incubated with mouse peroxidase anti-peroxidase (1:150) (M-PAP, Nordic Immunology). In the peroxidase reaction 0.025% (w/v) 3,3′-diaminobenzidine (DAB) was used as chromogen in the presence of 0.0005% (v/v) H_2O_2. Finally, sections were dehydrated and mounted. As a control for specificity the procedure was carried out as above, with the omission of the first antiserum.

2.11. Haematocrit levels

Immediately after blood puncture, subsamples were drawn into heparinized glass capillaries and centrifuged (13,600 *g*; 3 min) to assess haematocrit values. Results were rounded to the closest 0.5%.

2.12. Specific growth rate, feed intake and feed conversion ratio

On day 0 and day 42, fish were individually weighed to the nearest 1 g (Mettler PM 34 Delta range), to calculate the specific growth rate

(SGR) as follows:

$$SGR = (\ln(W_t) - \ln(W_0)) \times \frac{100}{t}$$

where SGR = specific growth rate (%/d), W_t = mean weight at day 42 (g), W_0 = mean weight at day 0 (g) and t = number of days.

In both experiments floating feed (Skretting R-2 15F) was given twice daily at 10 am and 2 pm until apparent satiation (no more feed taken for at least 5 min following administration of the feed). Feed loads per aquarium were recorded daily. All uneaten pellets were collected from each aquarium 1 h after each of the two daily feeding sessions. Feed loss per aquarium was calculated as the total number of uneaten feed pellets multiplied by 11.15 mg/pellet, the average weight of a pellet, determined by weighing 100 feed pellets. Daily feed intake per aquarium resulted from the difference between daily feed load and daily feed loss. To account for mortalities, daily feed intake per aquarium was divided by the number of fish in the aquarium, yielding the daily feed intake per fish in each aquarium. Cumulative daily feed intake per fish was calculated from the daily feed intake per fish in each aquarium. Total feed intake per fish (TFI) was determined as the cumulative feed intake at the last day of the experiment. Total feed intake per fish and biomass increase per fish were used to calculate feed conversion ratio (FCR) as follows:

$$FCR = \frac{TFI}{(W_t - W_0)}$$

where FCR = feed conversion ratio (g/g), TFI = total feed intake (g/fish), W_t = mean individual weight at day 42 (g) and W_0 = mean individual weight at day 0 (g).

2.13. Statistics

2.13.1. Physiological parameters

Physiological parameters are expressed as mean (SD) of the individual measurements per treatment. For each treatment, 24 fishes were sampled; in some instances less samples were analysed due to mortalities or because of insufficient plasma volume. When necessary, data were log-transformed to obtain residuals that were normally distributed and to obtain homogeneity of variance of residuals across treatment levels. Mean values for physiological parameters were tested for differences among the treatments using linear mixed models (REML) with treatments as fixed effects and aquarium as a random effect (F-tests with Kenward–Rogers approximation to the residual degrees of freedom (Kenward and Rogers, 1997)). Statistical analyses were performed in SAS 9.2 (SAS Institute Inc., Cary, North Carolina, USA). Only when significant treatment effects were detected, a least significant difference (LSD) post-hoc analysis was used to estimate the level of significance between mean values. For both REML and LSD analysis the fiducial limit was set at 5%. Linear regression analyses were performed with response variables as fixed effects and aquaria as random effects. In addition, F-tests with Kenward–Rogers approximation to the residual degrees of freedom were used (Kenward and Rogers, 1997). Pair-fed groups were not considered in regression analyses.

2.13.2. Feed intake and growth

Initial and final individual weight, specific growth rate (SGR) and feed conversion ratio (FCR) are presented as means per treatment (N = 2). Mean values per treatment were tested for significant differences among the treatments by one-way ANOVA. Only in case significant treatment effects were detected, a least significant difference (LSD) post-hoc analysis was used to estimate the level of significance between mean values.

Mean (N = 2) cumulative daily feed intake per treatment was tested for significant differences among the treatments by repeated measures ANOVA. Only in case significant treatment effects were detected, a post-hoc analysis (LSD for one-way ANOVA, Tukey for repeated measures ANOVA) was used to estimate the level of significance between mean values. All analyses were performed in SAS 9.2. For all analyses the fiducial limit was set at 5%.

2.13.3. Concentration–effect curves

NH_3 concentration–effect curves were fitted for specific growth rate (SGR) and total feed intake per fish (TFI) using a log-logistic model (Seefeldt et al., 1995). As a blank could not be included, the effects are expressed as absolute values. Curve-fitting was carried out with the Marquadt and Levenberg algorithm (Moré, 1978) as provided in the PRISM 4.00 software package (GraphPad Software, Inc.). The 10% effect concentrations (EC_{10}) and their 95% confidence limits were calculated (Miller and Miller, 2000).

3. Results

3.1. Plasma NH_4^+

Up to the second highest water NH_3 concentration (11.2 μM) the mean plasma NH_4^+ concentrations ranged between 614 and 762 μM without significant differences among treatments. However, at 18.6 μM water NH_3, the plasma NH_4^+ concentration had significantly increased to a nearly double level of 1399 μM compared to all other treatments (Table 3).

3.2. Plasma NO_3^-

Water NO_3^- concentration had a strong effect on plasma NO_3^- concentration. Plasma NO_3^- concentrations differed among all treatments (Table 4) and increased linearly with increasing water NO_3^- concentration ($[\text{Plasma } NO_3^-] = 0.23 * [\text{Water } NO_3^-] - 0.1(\text{mM})$; $P < 0.0001$). In pair-fed fish plasma NO_3^- levels (Table 4) were not affected.

Table 3
Mean (SD) values at the start (t = 0) and per NH_3 treatment for the end (t = 42 days) of the NH_3 experiment for plasma NH_4^+, plasma Cl^-, plasma osmolality and branchial Na^+/K^+-ATPase activity. Mean values with different superscripts are significantly different (REML, P values as shown). SD = standard deviation of mean values per treatment, n as indicated in the table. t = 0 values were not considered in the statistical analysis.

NH_3 treatment	Water NH_3 (μM)	Plasma NH_4^+ (μM)	n	Plasma Cl^- (mM)	n	Plasma osmolality (μOsmol/kg)	n	Na^+/K^+-ATPase activity (μmol Pi/h/mg protein)	n
1 — t = 0		716 (348)	11	153 (27)^{a}	22	304 (10)	23		
2 — Pair fed control	0.46	614 (175)^{a}	8	136 (24)^{a}	18	281 (9)^{ad}	20	1.44 (0.73)	12
3 — Control	0.90	657 (250)^{a}	12	150 (24)^{a}	13	296 (14)^{bc}	14	1.42 (0.45)	12
4 — NH_3	3.55	696 (222)^{a}	15	149 (19)^{a}	17	301 (18)^{b}	20	1.44 (0.38)	12
5 — NH_3	5.16	656 (131)^{a}	13	138 (22)^{a}	15	296 (9)^{bc}	17	1.40 (0.53)	12
6 — NH_3	7.12	759 (155)^{a}	16	149 (28)^{a}	17	283 (23)^{a}	21	1.17 (0.30)	11
7 — NH_3	11.2	762 (183)^{a}	15	140 (25)^{a}	18	286 (20)^{ac}	19	1.42 (0.51)	12
8 — NH_3	18.9	1399 (361)^{b}	13	107 (26)^{b}	12	270 (22)^{d}	16	1.52 (0.53)	11
P-value		0.04		0.0001		<0.0001		0.91	

Table 4
Mean (SD) values at the start (t = 0) and per NO_3^- treatment for the end (t = 42 days) of the NO_3^- experiment for plasma NO_3^-, plasma Cl^-, plasma osmolality and branchial Na^+/K^+-ATPase activity. Mean values with different superscripts are significantly different (REML, P values as shown). SD = standard deviation of mean values per treatment, n as indicated in the table. t = 0 values were not considered in the statistical analysis.

NO_3^- treatment	Water NO_3^-	Plasma NO_3^-	n	Plasma NO_3^- to water NO_3^- ratio	n	Plasma Cl^-	n	Plasma osmolality	n	Na^+/K^+-ATPase activity	n
	(mM)	(μM)				(mM)		(mOsmol/kg)		(μmol Pi/h/mg protein)	
1 — t = 0						121 (21)	20	320 (9)	20		
2 — Pair fed control	0.1	75 (27)^{a}	22	1.05 (0.38)^{a}	22	142 (24)	24	309 (13)^{ac}	24	1.2 (0.4)	8
3 — Control	0.1	88 (47)^{a}	19	0.90 (0.48)^{a}	19	152 (24)	24	303 (6)^{ab}	24	1.4 (0.3)	8
4 — NO_3^-	1.5	380 (52)^{ab}	20	0.26 (0.03)^{b}	20	149 (19)	23	303 (6)^{ab}	23	1.4 (0.7)	9
5 — NO_3^-	2.3	552 (90)^{ab}	18	0.24 (0.04)^{b}	18	141 (29)	24	299 (3)^{b}	24	1.2 (0.4)	9
6 — NO_3^-	3.7	820 (151)^{b}	20	0.22 (0.04)^{b}	20	152 (11)	22	308 (8)^{ac}	22	1.4 (0.3)	8
7 — NO_3^-	6.1	1378 (197)^{c}	19	0.23 (0.03)^{b}	19	150 (14)	24	308 (6)^{ac}	23	1.4 (0.7)	8
8 — NO_3^-	10.2	2136 (642)^{d}	14	0.20 (0.05)^{b}	14	133 (21)	22	307 (7)^{ac}	23	1.2 (0.3)	8
9 — NO_3^-	15.8	3493 (553)^{e}	19	0.22 (0.04)^{b}	19	144 (25)	23	303 (7)^{ab}	22	1.2 (0.4)	8
10 — NO_3^-	25.6	5993 (899)^{f}	21	0.23 (0.04)^{b}	21	135 (27)	24	312 (10)^{c}	23	0.8 (0.4)	8
P-value		<0.0001		<0.0001		0.64		0.06		0.40	

3.3. Plasma chloride and osmolality and branchial Na^+/K^+-ATPase activity

The increase in plasma NH_4^+ at the highest NH_3 concentration concurred with a significant decrease in plasma chloride to 107 mM. Plasma chloride ranged between 136 and 150 mM in all other NH_3 treatments without significant differences among these treatments (Table 3). For plasma osmolality significant differences were detected among NH_3 treatments (Table 3). No differences in plasma chloride concentration or osmolality were detected among NO_3^- treatments (Table 4).

No significant differences in branchial Na^+/K^+-ATPase activity were detected among NH_3 treatments (Table 3) or NO_3^- treatments (Table 4).

3.4. Haematocrit, methaemoglobin, plasma cortisol, glucose and lactate

No significant differences in haematocrit, plasma concentrations of cortisol, glucose and lactate were detected among NH_3 treatments (Table 5) and NO_3^- treatments (Table 6). Brown colouration of sampled blood, indicative of methaemoglobin formation, was not observed.

3.5. Gill morphology

Gill morphology (Fig. 1) was not affected by water NH_3, nor NO_3^-.

Table 5
Mean (SD) values at the start (t = 0) and per NH_3 treatment for the end (t = 42 days) of the NH_3 experiment for plasma cortisol, plasma glucose and plasma lactate concentrations and haematocrit. Mean values with different superscripts are significantly different (REML, P values as shown). SD = standard deviation of mean values per treatment, n as indicated in the table. t = 0 values were not considered in the statistical analysis.

NH_3 treatment	Water NH_3	Plasma cortisol	n	Plasma glucose	n	Plasma lactate	n	Haematocrit	n
	(μM)	(nM)		(mM)		(mM)		(%)	
1 — t = 0		14.3 (8.8)	23	5.63 (3.94)	22	7.56 (4.24)	23	23.1 (3.6)	22
2 — Pair fed control	0.46	12.6 (15.5)	21	7.94 (2.19)	20	4.11 (1.09)	20	32.3 (10.2)	21
3 — Control	0.90	14.3 (8.8)	15	5.99 (2.49)	16	5.34 (2.41)	14	40.8 (12.1)	17
4 — NH_3	3.55	12.4 (13.0)	20	6.65 (2.36)	20	5.73 (1.52)	20	31.8 (12.3)	20
5 — NH_3	5.16	11.3 (9.1)	18	7.17 (1.08)	17	5.77 (2.45)	17	33.7 (12.2)	23
6 — NH_3	7.12	17.7 (13.6)	21	7.15 (2.64)	21	4.51 (1.65)	18	38.0 (12.9)	23
7 — NH_3	11.2	16.0 (10.7)	19	7.27 (2.17)	19	4.34 (2.08)	18	37.3 (12.1)	19
8 — NH_3	18.9	10.4 (10.4)	18	6.99 (2.55)	18	5.30 (2.44)	16	29.5 (16.1)	19
P-value		0.87		0.85		0.63		0.08	

Table 6
Mean (SD) values at the start (t = 0) and per NO_3^- treatment for the end (t = 42 days) of the nitrate experiment for plasma cortisol, plasma glucose and plasma lactate concentrations and haematocrit. Mean values with different superscripts are significantly different (REML, P values as shown). SD = standard deviation of mean values per treatment, n as indicated in the table. t = 0 values were not considered in the statistical analysis.

NO_3^- treatment	Water NO_3^-	Plasma cortisol	n	Plasma glucose	n	Plasma lactate	n	Haematocrit	n
	(mM)	(nM)		(mM)		(mM)		(%)	
1 — t = 0		13 (22)	20	5.4 (2.8)	22	7.5 (3.5)	22		12
2 — Pair fed control	0.1	134 (106)	24	7.5 (1.9)	24	7.3 (3.3)	13	37 (4)	24
3 — Control	0.1	210 (150)	23	7.5 (1.6)	24	6.8 (2.6)	20	36 (4)	24
4 — NO_3^-	1.5	119 (123)	23	7.5 (1.9)	23	7.0 (2.5)	18	38 (7)	24
5 — NO_3^-	2.3	166 (162)	24	7.3 (1.8)	24	5.4 (3.1)	19	37 (1)	24
6 — NO_3^-	3.7	123 (132)	22	7.7 (1.9)	24	8.0 (3.0)	18	31 (6)	24
7 — NO_3^-	6.1	186 (107)	24	7.9 (1.3)	24	8.2 (4.0)	18	36 (5)	24
8 — NO_3^-	10.2	155 (123)	22	7.5 (1.6)	24	6.8 (3.5)	14	36 (2)	24
9 — NO_3^-	15.8	115 (132)	21	7.5 (2.4)	24	7.3 (3.1)	15	38 (5)	23
10 — NO_3^-	25.6	47 (31)	23	7.9 (1.5)	24	8.6 (3.7)	17	35 (6)	24
P-value		0.76		0.99		0.99		0.86	

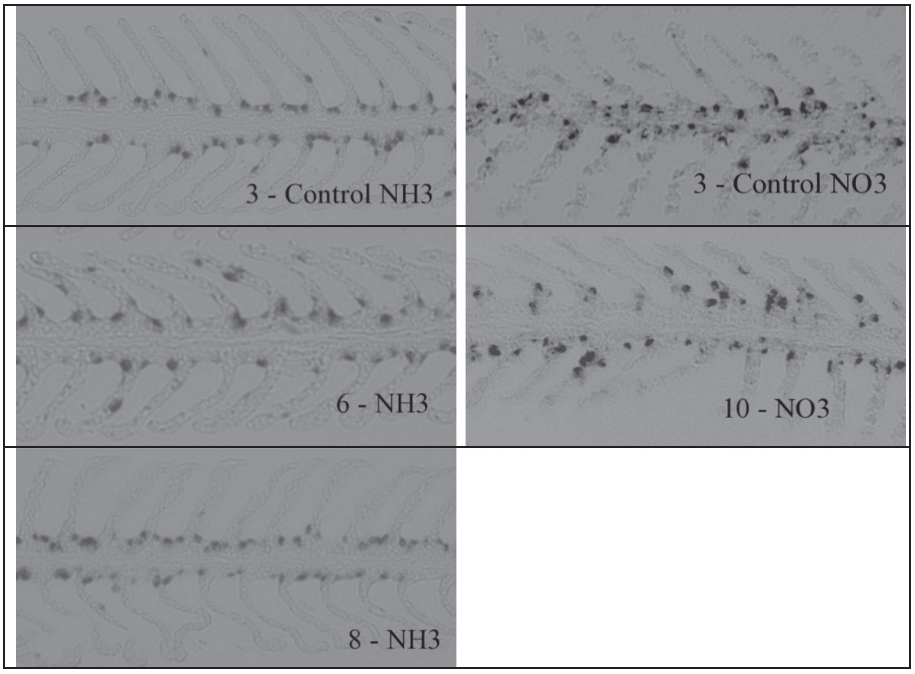

Fig. 1. Histology of gill epithelium immunohistochemically stained for Na^+/K^+-ATPase-rich cells (chloride cells) of the 0.90 μM (3 — Control), 7.1 μM (6 — NH_3) and 18.9 μM (8 — NH_3) NH_3 treatment groups and the 0.1 mM (3 — Control NO_3^-) and 25.6 mM (10 — NO_3^-) NO_3^- treatment groups (200× magnification).

3.6. Feed intake, specific growth rate, feed conversion ratio and mortality

In the NH_3 experiment a total of 19 out of 168 fish had died across NH_3 treatments, resulting in a survival rate ranging between 71 and 100% among treatments. Mortality was probably a consequence of low feed intake as mortalities occurred mainly towards completion of the NH_3 experiment and, without exception, dead fish appeared emaciated (data not shown). Mortality could not be related to the NH_3 treatments (Table 7), leaving the underlying reason for emaciation unclear.

NH_3 exposure had a strong effect on total feed intake (TFI) and specific growth rate (SGR). For both TFI and SGR differences were detected among NH_3 treatments (Table 7). At the highest NH_3 concentration (18.9 μM) the TFI had decreased by 69% and SGR by 75% compared to control. The differences in TFI developed over time (Fig. 2). Mean feed conversion ratios (FCR) did not differ among NH_3 treatments (Table 7).

No fish died in the NO_3^- experiment. Differences in final weight, total feed intake (TFI), specific growth rate (SGR) and feed conversion ratio (FCR) were not detected among NO_3^- treatments (Table 8).

3.7. EC_{10} for total feed intake and SGR

The concentration–effect curves for TFI and SGR in relation to the water NH_3 concentration (Fig. 3A and B), yield an EC_{10} for NH_3 of 7.1 μM (0.1 mg NH_3–N/L), with a 95% confidence interval from 5.1 to 9.8 μM when read against TFI. For SGR, a somewhat lower EC_{10} for NH_3 of 5.7 μM (0.08 mg NH_3–N/L), with a 95% confidence interval from 3.4 to 9.7 μM was calculated.

4. Discussion

4.1. NH_3 experiment

Juvenile pikeperch (*S. lucioperca*) successfully control plasma NH_4^+ up to a water NH_3 concentration of 11.2 μM. However, water NH_3 levels below 11.2 μM lead to reduced feed intake and growth and thus appear superior parameters to assess NH_3-tolerance.

Table 7
Mean (SD) values per NH_3 treatment (N = 2) in the NH_3 experiment for initial weight, final weight, total feed intake (TFI), specific growth rate (SGR) and feed conversion ratio (FCR). Mean values with different superscripts are significantly different (one-way ANOVA[a], P values as shown). SD = standard deviation of mean values per treatment.

NH_3 treatment	Water NH_3 (μM)	Initial weight (g)	Final weight (g)	TFI (g/fish)	SGR (%BW/d)	FCR	Survival (%)
1 — t = 0		16.1 (0.57)					
2 — Pair fed control	0.46	17.3 (0.41)	25.3 (0.20) ^{ab}	7.5 (0.21)^{a}	0.95 (0.04)^{a}	0.94 (0.00)	92 (0.0)
3 — Control	0.90	18.7 (3.19)	53.4 (12.6)^{c}	21.7 (0.55)^{bc}	2.61 (0.17)^{b}	0.65 (0.16)	71 (18)
4 — NH_3	3.55	18.2 (0.58)	46.9 (1.37)^{cd}	23.0 (1.78)^{bc}	2.36 (0.01)^{bc}	0.80 (0.04)	88 (6)
5 — NH_3	5.16	17.7 (0.04)	46.0 (4.89)^{cd}	24.1 (1.73)^{b}	2.38 (0.26)^{bc}	0.86 (0.09)	96 (6)
6 — NH_3	7.12	17.9 (0.38)	40.4 (0.62)^{cd}	19.8 (1.04)^{c}	2.04 (0.09)^{cd}	0.88 (0.01)	100 (0)
7 — NH_3	11.2	18.1 (0.45)	36.7 (6.16)^{ad}	15.4 (0.13)^{d}	1.75 (0.36)^{d}	0.87 (0.26)	88 (18)
8 — NH_3	18.9	17.3 (0.27)	22.5 (1.07)^{b}	6.7 (0.32)^{a}	0.65 (0.08)^{a}	1.31 (0.27)	88 (6)
P-value		0.91	0.007	<0.0001	<0.0001	0.07	0.27

[a] For TFI repeated measures ANOVA was used.

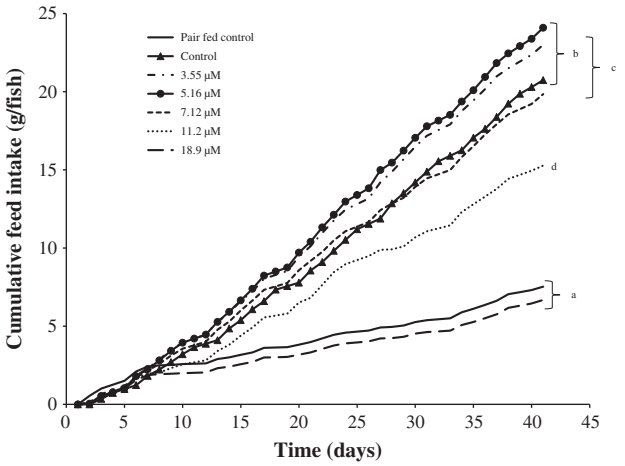

Fig. 2. Mean (n = 2) cumulative feed intake of juvenile pikeperch exposed to ammonia. For series marked with different letters the cumulative feed intake resulted in significantly different total feed intake (repeated measures ANOVA, $P < 0.05$).

4.1.1. Plasma NH_4^+, feed intake and growth

Fish produce ammonia as main end product of the catabolism of ingested proteins (Handy and Poxton, 1993). Ammonia then appears in the plasma compartment, which shows postprandial peaks (Wicks and Randall, 2002a). Ammonia toxicity is avoided in fish by up-regulating muscle glutamine synthetase activity (Wicks and Randall, 2002b) and by excreting ammonia across the gills to the water (Wilkie, 2002). High external (water) ammonia leads to an influx of ammonia in plasma and tissues (Wright et al., 2007). To avoid toxic plasma ammonia levels during an influx of external ammonia, fish reduce their own ammonia production by reduction of food intake (Randall and Tsui, 2002). This ammonia defence mechanism explains the reduced feed intake we observed in ammonia exposed pikeperch. Differences in feed intake showed up more or less instantaneously at the two highest NH_3 levels (11.2 and 18.9 μM), resulting in significant differences in total feed intake among treatments at the end of the experiment. We conclude from this observation that the reduced growth in pikeperch in response to NH_3 is mainly an effect of reduced feed intake, which corresponds to previous observations on NH_3 exposed turbot (*Scophthalmus maximus*) (Person-Le Ruyet et al., 1997) and African catfish (*Clarias gariepinus*) (Schram et al., 2010).

A higher ability of fish to cope with high external ammonia has been related to lower plasma ammonia concentrations in these fish (Wicks and Randall, 2002a). The basal plasma NH_4^+ concentrations of around 650 μM observed in pikeperch seem high compared to values reported for other fish species (~160 μM NH_4^+ in African catfish (Schram et al., 2010); ~200 μM T_{amm} in Atlantic salmon (*Salmo salar*) (Knoph and Thorud, 1996); ~300 μM T_{amm} in European seabass (*Dicentrarchus labrax*) (Lemarié et al., 2004)), may reflect an adaptation to piscivory, and may be related to increased tolerance to NH_4^+ through protonation of NH_3 in the plasma compartment.

We advise to set the water NH_3 threshold concentration for growth at 3.4 μM, the lower limit of the 95% confidence interval of the EC_{10} we calculated for growth. This indeed classifies juvenile pikeperch as significantly more sensitive to water ammonia levels than for instance African catfish (EC_{10} of 24 μM). Atlantic salmon appears more sensitive than pikeperch, with growth being affected already above 1.4 μM NH_3 (Arillo et al., 1981); also, basal plasma total ammonium values in Atlantic salmon are relatively low (~200 μM T_{amm}, Knoph and Thorud, 1996). From that perspective pikeperch seem relatively robust and tolerate high water ammonia through the ability to maintain acceptable plasma NH_4^+ values over a rather wide range of water ammonia levels. The prediction would be that this tolerance increases at lower water temperatures assuming similar chemistry of NH_3 in water and blood plasma (Emerson et al., 1975). In Atlantic salmon (Knoph and Thorud, 1996) and several other marine species such as European seabass, gilthead seabream (*Sparus aurata*) and turbot (Person-Le Ruyet et al., 1997) this ability seems absent as plasma ammonia has been observed to increase linearly with ammonia in the water. Unfortunately the possibilities to explore the relation between basal plasma ammonia and ammonia sensitivity are limited as data on plasma ammonia and detailed threshold concentrations for chronic NH_3 exposure of fish are scarce.

The ability of pikeperch to buffer plasma NH_3 to NH_4^+ at higher water NH_3 is limited yet considerable as the plasma NH_4^+ concentration in pikeperch exposed to 18.9 μM NH_3 in the water doubled to almost 1.4 mM compared to control values. Apparently a threshold is surpassed between 11.2 and 18.9 μM NH_3 above which pikeperch can no longer maintain low plasma NH_4^+ concentrations.

4.1.2. Plasma osmolality, plasma chloride, Na^+/K^+-ATPase activity and gill morphology

Freshwater fish continuously lose ions via diffusion across the mucous epithelium of gills and skin to the surrounding less saline water (Evans et al., 2005). For homeostasis of bodily fluids freshwater fish tightly regulate plasma osmolality by active Na^+ and Cl^- uptake (McDonald and Wood, 1981). Hypochloremia was detected in pikeperch exposed to 18.9 μM NH_3 (concurrent with a significant increase in plasma NH_4^+) revealing a disturbance of chloride-homeostasis and interference of plasma NH_4^+ with chloride handling. Branchial and intestinal chloride uptake from water and food and renal reabsorption are key to chloride homeostasis in freshwater fish (Fuentes et al., 1997). It would seem then that the significant decrease in food intake seen at high water NH_3 also interferes with chloride regulation in this fish. Further, significant differences in osmolality were detected among NH_3

Table 8
Mean (SD) values per NO_3^- treatment (N = 2) in the NO_3^- experiment for initial weight, final weight, total feed intake (TFI), specific growth rate (SGR) and feed conversion ratio (FCR). SD = standard deviation of mean values per treatment. No significant differences were detected among treatments (one-way ANOVA[a], P-values as shown).

NO_3^- treatment	Water NO_3^- (mM)	Initial weight (g)	Final weight (g)	TFI (g/fish)	SGR (%BW/d)	FCR
1 — t = 0		26.4 (0.3)				
2 — Pair fed control	0.1	26.6 (0.5)	67.9 (3.3)	34.7 (2.1)	2.29 (0.16)	0.81 (0.02)
3 — Control	0.1	27.5 (2.0)	72.5 (0.5)	35.2 (1.1)	2.31 (0.16)	0.78 (0.00)
4 — NO_3^-	1.5	26.3 (3.7)	70.4 (2.1)	35.4 (1.0)	2.35 (0.26)	0.80 (0.01)
5 — NO_3^-	2.3	27.4 (0.0)	70.8 (3.5)	35.8 (1.4)	2.26 (0.12)	0.82 (0.03)
6 — NO_3^-	3.7	27.7 (0.3)	65.7 (3.7)	30.7 (3.1)	2.05 (0.11)	0.81 (0.01)
7 — NO_3^-	6.1	27.4 (0.3)	72.8 (3.7)	35.5 (1.7)	2.33 (0.10)	0.78 (0.02)
8 — NO_3^-	10.2	27.5 (0.2)	70.2 (4.2)	34.5 (2.7)	2.23 (0.13)	0.81 (0.01)
9 — NO_3^-	15.8	25.4 (0.5)	68.2 (6.1)	33.8 (3.8)	2.35 (0.17)	0.79 (0.02)
10 — NO_3^-	25.6	28.2 (1.8)	69.5 (3.2)	33.6 (0.5)	2.15 (0.04)	0.82 (0.02)
P-value		0.58	0.81	0.44	0.46	0.25

[a] For TFI repeated measures ANOVA was used.

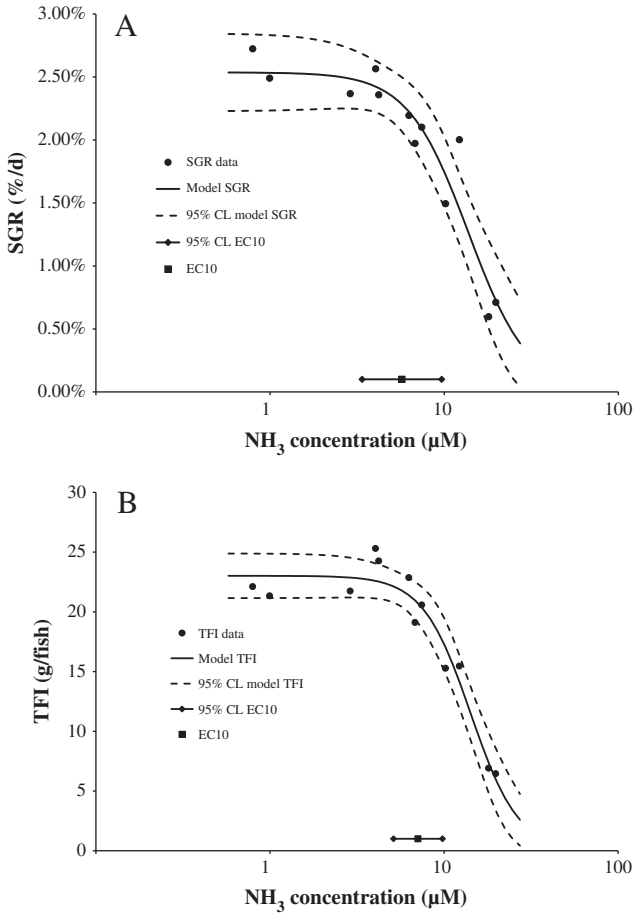

Fig. 3. Concentration–effect curves for specific growth rate (SGR, A) and total feed intake ("TFI data") in relation to the water NH_3 concentration. $SGR = 0.025 - 0.025 / (1 + 10^{(-(\log[NH_3] - 1.14) / 0.40)})$ ($r^2 = 0.90$) and $TFI = 23.1 - 23.1 / (1 + 10^{(-(\log[NH_3] - 1.15) / 0.32)})$ ($r^2 = 0.94$). EC_{50} for SGR = 13.7 µM NH_3, EC_{50} for TFI = 14.2 µM NH_3. CL = confidence limit.

treatments. Significantly reduced osmolality as compared to the control treatment was also observed in pair-fed controls. Thus the decrease in osmolality may indeed be an effect of reduced feed intake rather than NH_3 exposure per se.

At high ambient ammonia several active NH_4^+ excretion pathways could facilitate ammonia efflux (Wright and Wood, 2009). Na^+/K^+-ATPase is a driving force in active NH_4^+ excretion when internal NH_4^+ (replacing K^+ on the enzyme) is exchanged for waterborne Na^+ (reviewed by Heisler, 1984; Evans, 1987; Evans et al., 2005). Increased branchial Na^+/K^+-ATPase activity in response ammonia exposure has been described for several fish species (Alam and Frankel, 2006; Sinha et al., 2012). However, in pikeperch branchial Na^+/K^+-ATPase activity did not increase in response to increased ambient NH_3 nor plasma NH_4^+. An extensive (re-)analysis of NH_3 chemistry at the (sub-)cellular level and consideration of NH_3 transporter (Rhesus) proteins (Nakada et al., 2007) seem indicated. At least four Rhesus protein species were described in branchial epithelium of zebrafish (Braun et al., 2009), but analysis of these is beyond the scope of this study.

There appears to be no consensus on the effect of ammonia on fish gill morphology. In the past gill hyperplasia has been proposed as a common indicator for ammonia toxicity in fish (Smith and Piper, 1975 in Mitchell and Cech, 1983; Redner and Stickney, 1979 in Mitchell and Cech, 1983) based on various ammonia toxicity studies presenting evidence of gill epithelial damage (citations in Mitchell and Cech, 1983). However, no evidence of gill damage could be detected in ammonia exposed rainbow trout (*O. mykiss*) (Smart, 1976), Dover sole (*Solea solea*) and turbot (*S. maximus*) (Alderson, 1979). The validity of attributing gill hyperplasia to ammonia alone was questioned by Mitchell and Cech (1983) once they showed that gill hyperplasia was absent in ammonia exposed channel catfish (*Ictalurus punctatus*), except when low levels of chlorine compounds, residuals from municipal water treatment, were present next to ammonia. Indeed, for many ammonia toxicity studies in the past the presence of chlorine compounds cannot be excluded. Gill damage observed in past ammonia toxicity studies should however not be automatically attributed to chlorine rather ammonia exposure as in African catfish gill morphology gradually changed with increasing NH_3 exposure concentration in water that had not been chlorinated (Schram et al., 2010).

Morphological changes of the gills may be interpreted as adaptations to increase the diffusion distance between the water and the blood flow, reducing the permeability of the gills and subsequently the influx of NH_3. No clear effects of ammonia exposure on gill morphology were observed in pikeperch. The absence of such a morphological response in pikeperch gills surprised us as pikeperch were clearly affected by the highest NH_3 exposure concentration (18.9 µM). However, the absence of morphological changes in the gills is not exceptional and has been previously reported in other ammonia exposed fish species (see above).

4.1.3. Stress physiology

Plasma cortisol levels below 50 ng/mL or 138 nM are considered as stress free levels (typical basal levels for common carp (*C. carpio*) below 15 nM; Metz et al., 2005). Increases up to 166 nM are generally referred to as a mild response, while rapid increases above 276 nM are generally considered to reflect a severe stress response (Wendelaar Bonga, 1997). The stress and energy metabolite parameters (plasma cortisol, glucose and lactate) in pikeperch were not affected by NH_3 exposure. Plasma cortisol values found in this study (10–18 nM) are very much lower than values reported in other studies on pikeperch (>700 nM, Falahatkar et al., 2012; 124–180 nM, Sarameh et al., 2012) and probably the best representation of basal cortisol levels in pikeperch to date. Plasma glucose and lactate levels observed in the NH_3 experiment are slightly higher than control values reported in other pikeperch studies (5 mM lactate, 4.5–6 mM glucose, Falahatkar et al., 2012) but lie in the range that can be considered normal. We conclude from these observations that ammonia even at its highest exposure concentration apparently did not impose distress.

4.2. NO_3^- experiment

NO_3^- exposed juvenile pikeperch accumulated nitrate in the plasma compartment, but no effects on physiology and growth were detected even at water nitrate as high as 26 mM, commensurate with the notion that the end product of the nitrogen waste cycle is relatively harmless to fish. The high NO_3^- tolerance allows for low water exchange of RAS used for juvenile pikeperch culture. However it should be noted that, next to apparently relatively harmless NO_3^-, other, potentially harmful compounds may then accumulate in the rearing water.

4.2.1. Plasma NO_3^-, feed intake and growth

Nitrate can become toxic to fish (Camargo et al., 2005). Chronic exposure to high water nitrate leads to nitrate accumulation in the plasma compartment and reduced feed intake and growth in African catfish (Schram et al., in press). In pikeperch plasma NO_3^- concentrations increased linearly with increasing external NO_3^- concentrations, seemingly unaffected by the differences in sodium concentration and conductivity among treatments. Plasma NO_3^- increased in a very similar manner to what we observed in NO_3^--exposed African catfish (Schram et al., in press). Surprisingly, whereas African catfish showed reduced growth and feed intake upon nitrate exposure, pikeperch was refractory to this treatment. Clearly species-specific differences in NO_3^- toxicity exist and are not related to differences in the capability to maintain low plasma NO_3^- when external NO_3^- is high. Instead the internal NO_3^-

handling seems much more important. Differences in sodium concentration and conductivity arising from the sodium nitrate addition to the aquaria apparently did not affect NO_3^- accumulation in the plasma given the linear increase in plasma NO_3^- with increasing external NO_3^- concentrations.

The growth performance of the pikeperch in our NO_3^- experiment (overall mean (SD) specific growth rate of 2.26 (0.15)%/d) corresponds well to growth performances of similar sized pikeperch at the same water temperature in commercial pikeperch farming (Vestergaard, personal communication).

Previously observed molar ratios (plasma:water) of approximately 0.2 in nitrate exposed rainbow trout (Stormer et al., 1996) and African catfish (Schram et al., in press) suggest that the integument forms a significant barrier to waterborne nitrate. The molar ratios we observed in pikeperch range from 0.23 to 0.26 and are in good agreement with these observations, suggesting similar nitrate handling in rainbow trout, African catfish and pikeperch. Daily plasma sampling of catheterized, nitrate exposed rainbow trout over a period (8 days) revealed that an apparent chemical equilibrium was reached within a day (Stormer et al., 1996). We have no data that describe the time-kinetics of plasma nitrate levels in pikeperch. However, considering Stormer et al.'s (1996) observations on rainbow trout it seems unlikely that after 42 days of nitrate exposure, nitrate further accumulates in the plasma to levels that are eventually not tolerated by pikeperch. We therefore consider the current experiment representative for chronic nitrate exposure. The millimolar plasma NO_3^- concentrations that are apparently tolerated by pikeperch support the notion that NO_3^- is not very toxic to fish and may be considered an end product and stable non-toxic form of nitrogenous waste.

4.2.2. NO_3^- effects on physiology and gill morphology

High levels of NO_3^- in the water did not affect plasma osmolality and plasma chloride. NO_3^- exposure did not affect branchial Na^+/K^+-ATPase activity. Apparently a significant hypernitratemia does not alter the activity if this enzyme. This corresponds to our observation that the number of Na^+/K^+-ATPase rich chloride cells was not affected by NO_3^- exposure.

NO_3^- exposure did not cause morphological changes nor anomalies of the branchial epithelium of pikeperch. The results taken jointly indicate that high levels of NO_3^- do not affect permeability of the gills, neither to water or ionic species central to osmotic homeostasis, nor to NO_3^- itself (Stormer et al., 1996) as we conclude from the linear mild increase in molar ratios for NO_3^- in water and plasma; clearly, NO_3^- is not very toxic for this species.

Considering the mildly elevated plasma cortisol values (just over 100 nM) observed in all NO_3^- treatments, it seems likely that treatment effects (if any) on plasma cortisol were overridden by cortisol release due to sampling procedures. However, the normal values observed for plasma glucose and plasma lactate in pikeperch suggest that NO_3^- exposure did not chronically stress the fish (Wendelaar Bonga, 1997).

4.3. Effect of fish size and life-stage on NH_3 and NO_3^- threshold concentrations

Toxicity of nitrogenous compounds to fish has been shown to vary with size and life-stage. NH_3 sensitivity was shown to decrease with age and size during the early ontogeny of chub (*Leuciscus cephalus*) (Gomulka et al., 2011). NH_3 tolerance in rainbow trout has been reported to increase as fish develop through the larval stages, to peak at juvenile and yearling stages and decrease thereafter (Thurston and Russo, 1983).

NO_3^- sensitivity may decrease with increasing size of aquatic invertebrates and amphibians (Camargo et al., 2005). Specific for fish it has been shown that the early life-stages of lake trout (*Salvelinus namaycush*) and lake whitefish (*Coregnus clupeoformisi*) are much more sensitive to NO_3^- than the juveniles of these species (McGurk et al., 2006). Information on NO_3^- sensitivity in relation to body size beyond the juvenile stages of fish is lacking. However, nitrite sensitivity has been reported to increase with increasing size of Nile tilapia (*Oreochromis niloticus*) (Atwood et al., 2001) and fathead minnow (*Pimephales promelas*) (Palachek and Tomasso, 1984).

This information jointly taken, it is quite likely that NH_3 and NO_3^- sensitivity of pikeperch varies with size and life-stage. Consequently the here reported NH_3 and NO_3^- threshold concentrations should be cautiously applied outside the size range (17–25 g) we tested. Safe levels for early life-stages are probably lower than the here reported NH_3 and NO_3^- threshold concentrations, as it is reasonable to consider the early life-stages of pikeperch to be more sensitive than the juveniles we tested. There appear to be no indications to consider larger pikeperch to be more or even equally tolerant to NH_3 and NO_3^- as juveniles. The here reported thresholds could be cautiously applied to larger pikeperch than tested, keeping in mind that safe levels are in fact possibly lower. Note in this respect that reduced feed intake is a strong indicator for too high ammonia and too high nitrate.

5. Conclusions

Juvenile pikeperch chronically exposed to NH_3 as high as 11.2 μM NH_3 did not show major physiological disturbances. However, feed intake and growth decreased already at very much lower NH_3 concentrations: the EC_{10} were found to be 5.7 μM for SGR and 7.1 μM for TFI. Feed intake and growth are thus good and easily assessed indicators for negative effects of high NH_3 on pikeperch. Considering the lower limit of the 95% confidence interval of the lowest EC_{10} value, the NH_3 threshold concentration for juvenile pikeperch should be set at 3.4 μM (0.05 mg NH_3–N/L).

Juvenile pikeperch chronically exposed to the highest NO_3^- test concentration (25.6 mM) did not show major physiological disturbances or reduced growth performance. The threshold concentration for chronic NO_3^- exposure of juvenile pikeperch thus seems to lie outside the NO_3^- range investigated in the current experiment. We propose to use the highest test concentration that (still) showed no significant effect as a safe threshold concentration for NO_3^-: 25.6 mM (358 mg NO_3^-–N/L).

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