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The impact of elevated water nitrate concentration on physiology, growth and feed intake of African catfish *Clarias gariepinus* (Burchell 1822)

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Abstract

The nitrate threshold concentration in rearing water of African catfish (*Clarias gariepinus*) was assessed. Female African catfish with an initial mean (SD) weight of 154.3 (7.5) g were exposed to 0.4 (Control), 1.5, 4.2, 9.7 and 27.0 mM nitrate for 42 days. Mean (SD) plasma concentrations of nitrate increased from 71 (29) to 6623 (921) μM at the highest ambient nitrate level. Mean (SD) plasma nitrite concentration ranged from 1.2 (0.5) to 7.9 (9.0) μM. Haematocrit, plasma concentrations of non-esterified fatty acids (NEFA), cortisol, glucose, lactate, osmolality, gill morphology and branchial Na^+/K^+-ATPase activity were not affected. Feed intake and specific growth rate were significantly reduced at the highest nitrate concentration. We advise not to exceed a water nitrate concentration of 10 mM (140 mg L^{-1} NO_3-N) to prevent the risk of reduced growth and feed intake in African catfish aquaculture.

Keywords: African catfish, nitrate toxicity, specific growth rate, feed intake, stress physiology, recirculating aquaculture systems

Introduction

Aquatic organisms risk exposure to toxic levels of nitrate in their natural environment due to agricultural application of fertilizers (Bouchard, Williams & Surampalli 1992) and in intensive aquaculture when recirculation systems (RAS) are used (Van Rijn 2010). Nitrate is toxic to teleosts. Camargo, Alonso and Salamanca (2005) suggested, in analogy to the mechanism of nitrite toxicity (Eddy & Williams 1987; Williams, Glass & Heisler 1993), that nitrate toxicity is due to conversion of haemoglobin to methaemoglobin and the inherent loss of oxygen carrier capacity. Uptake of nitrate via the integument from the water by aquatic animals is low compared to that of nitrite. In fish, this is attributed to an apparent low branchial permeability to nitrate (Stormer, Jensen & Rankin 1996); similar conclusions were reached for gill-breathing crustacean species (Jensen 1996; Cheng & Chen 2002). Nitrate is therefore considered to be less toxic than nitrite. Acute and chronic effects of nitrate exposure were studied in freshwater channel catfish (*Ictalurus punctatus*): the lethal nitrate concentration (96 h LC_{50} at 26°C) was as high as 105 mM (Colt & Tchobanoglous 1976). No obvious adverse effects were observed during 164 days exposure of channel catfish to 6.4 mM nitrate (Knepp & Arkin 1973).

African catfish (*Clarias gariepinus*) is commercially farmed in intensive RAS in the Netherlands. In RAS ammonia is converted to nitrate in aerobic biological filters. Nitrate accumulates in the system (Bovendeur, Eding & Henken 1987; Eding, Kamstra, Verreth, Huisman & Klapwijk 2006) and

fish farmed in RAS may be chronically exposed to nitrate levels ranging from 7 to 70 mM (100–1000 mg L^{-1} N) depending on RAS design and management (Van Rijn 2010). African catfish are typically exposed to nitrate levels around 7 mM (Verreth & Eding 1993). The effects of this chronic nitrate exposure on African catfish physiology are not known. We exposed African catfish to increased water nitrate levels for 42 days and the nitrate threshold concentration was assessed.

Materials and methods

Experimental conditions

Female African catfish *(Clarias gariepinus)* were obtained from Fleuren-Nooijen BV, Someren, the Netherlands. Fish (n = 208) were randomly divided over sixteen 30-L rectangular glass, dark covered, tanks and acclimatized to the experimental tanks for 7 days. At the start of the 42 day experiment, the overall initial mean (SD) individual weight was 154.3 (7.5) g. The resulting mean stocking density was 66.9 kg m^{-3}, well below fish densities found at commercial farms for this size class (100–300 kg m^{-3}, Van de Nieuwegiessen, Olwo, Khong, Verreth & Schrama 2009).

The treatment of the fish was in accordance with Dutch law concerning animal welfare, as tested by the ethical committee for animal experimentation of Wageningen UR Livestock Research (number 2011015.c).

The experiment consisted of eight duplicated treatments. Treatments were assigned randomly to the tanks. Treatment 1 was included to collect blood and plasma at the start of the experiment (t = 0). Fish in treatments 2–6 were exposed to one of five different nitrate concentrations in the water: 0.4 (control), 1.5, 4.2, 9.7 and 27.0 mM. Fish in treatment 7 (pair-fed) were kept at 0.4 mM nitrate and pair-fed to the fish kept in 27.0 mM nitrate (treatment 6) to discriminate between effects caused by low feed intake and exposure to a high nitrate concentration in the water. Fish in treatment 8 (chloride) were exposed to high nitrate (22.5 mM) in the presence of sodium chloride (2.4 g L^{-1}) to evaluate a potential attenuating effect of chloride (and sodium) on nitrate toxicity, similar to nitrite toxicity (Eddy, Kunzlik & Bath 1983).

During acclimatization and experimental periods, all tanks were supplied with local tap water via a header tank at a flow of 185 L d^{-1}. Experimental nitrate concentrations were realized by infusion of $NaNO_3$ stock solutions prepared in tap water (Table 1), which were pumped into the tanks by a peristaltic pump (Watson Marlow 505 S, Rotterdam, the Netherlands) at a flow of 4.75 L d^{-1} per tank. Each tank was equipped with an air stone to guaranty good mixing of the infused stock solution with the tank water. Flow rates were monitored daily and adjusted when necessary to reach the desired nitrate concentrations. Nitrate concentrations were gradually increased to the designated concentrations during the first 4 days of the experimental period. Fresh stock solutions (Table 1) were prepared daily during the first 11 days of the experimental period and in this period nitrate, nitrite and total ammonia (NH_4^+-N plus NH_3-N) concentrations were monitored daily (Spectroquant cell tests for NO_3^--N, NO_2^--N and NH_4^+-N, Merck, Darmstadt, Germany, in a Hach Lange DR2800 spectrophotometer, Düsseldorf, Germany). During the remainder of the experimental period, fresh stock solutions were prepared weekly and nitrate, nitrite (Table 1) and total ammonia concentrations (0.33–0.57 mM) monitored twice per week. Water temperature (25.4–25.6°C), pH (Table 1) and dissolved oxygen concentrations (0.27–0.39 mM) were monitored daily prior to feeding in all tanks (Hach Lange HQ 40D multimeter, Loveland, CO, USA) throughout the entire experiment.

Blood and plasma sampling

One day before exposure to nitrate started (day 0), fish in treatment 1 were sampled. After 42 days exposure to nitrate, the fish from the seven remaining treatments were sampled (13 fish per tank). Fish were rapidly netted and anaesthetised in 0.1% (v/v) 2-phenoxyethanol (Sigma, St. Louis, MO, USA). Within 2 min, blood (2 × 1.0 mL) was taken by puncture of the caudal vessels by the use of a heparinized syringe fitted with a 25-gauge needle. One 50 μL aliquot was used for the haematocrit measurement, the remainder was immediately centrifuged for 10 min (14 000 ***g***, 4°C) and the plasma so obtained stored at −20°C until further analyses.

Plasma nitrate and nitrite concentration

NO_x (the sum of NO_2^- and NO_3^-) was measured with the nitrate/nitrite colorimetric assay kit from

Table 1 Composition of the treatment-specific stock solutions, the predicted* nitrate, sodium and chloride concentrations and the mean (SD) values for measured nitrate and nitrite concentrations in the tanks for all treatments

Treatment	$NaNO_3$ in stock (g L^{-1})	NaCl in stock (g L^{-1})	Predicted tank $[NO_3^-]$ (mM)	Predicted tank $[Na^+]$ (mM)	Predicted tank $[Cl^-]$ (mM)	Predicted tank salinity (g L^{-1})	$[NO_3\text{-}N]$ (mg L^{-1})	$[NO_3\text{-}N]$ (mM)	$[NO_2\text{-}N]$ (μM)	pH range
2-control	0	0	0	0	0	0	5.5 (0.31)	0.39 (0.02)	4.6 (1.6)	7.30–8.06
3	3.5	0	1.07	1.07	0	0.02	20.5 (0.51)	1.46 (0.04)	8.5 (2.9)	7.35–7.75
4	10.6	0	3.21	3.21	0	0.05	59.0 (8.86)	4.21 (0.63)	7.0 (3.6)	7.36–7.92
5	31.9	0	9.64	9.64	0	0.14	135.8 (10.20)	9.69 (0.73)	7.3 (2.6)	7.35–7.76
6	95.6	0	28.90	28.90	0	0.41	378.8 (32.80)	27.04 (2.34)	10.2 (1.9)	7.40–7.89
7-pair-fed	0	0	0	0	0	0	5.6 (0.23)	0.40 (0.02)	7.1 (3.3)	7.39–7.69
8-chloride	95.6	77.8	28.90	63.20	34.2	2.40	315.0 (25.30)	22.49 (1.80)	3.5 (1.3)	7.55–7.99

*Based on equal flow rates per tank of 4.75 L d^{-1} for the stock solutions and 185 L $d^{-1}$for the tap water flow.

Cayman Chemical Company (Ann Arbor, Michigan, USA). Prior to measurement, plasma samples were ultrafiltrated using Millipore Ultra-free MC centrifugal filter device (0.1 μm pore size) to reduce background absorbance due to the presence of haemoglobin and improve colour formation using the 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 Nitrate reductase mixture. Fifty μL of the first Griess reagent (R1) followed by the second Griess reagent (R2) was added and absorbance was read at 530 nm using a Wallac 1420 VICTOR^2 counter (Turku, Finland). The nitrite (NO_2) fraction within NO_x was measured using 100 μL of undiluted plasma together with the Griess reagents combination. The NO_3^- fraction was calculated as the difference between the plasma NO_x and the plasma NO_2^- concentrations.

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

Plasma concentration of cortisol was determined by radioimmunoassay as described in detail elsewhere (Metz, Geven, Van den Burg & Flik 2005). Plasma osmolality was measured using a cryoscopic osmometer (Osmomat 030; Gonotec, Germany). Plasma concentrations of glucose, lactate were measured with commercially available enzymatic kits adapted to 96 wells plates as described recently (Schram, Roques, Abbink, Spanings, De Vries, Bierman, Van de Vis & Flik 2010). Branchial Na^+/K^+-ATPase activity was measured as described elsewhere (Metz, Van den Burg, Wendelaar Bonga & Flik 2003).

Plasma NEFA concentration

Plasma non-esterified fatty acid (NEFA) concentration was measured with a commercial kit (Wako Chemicals GmbH, Neuss, Germany), with a protocol adapted to a 96-well microplate. Four μL of sample or standard (0, 0.25, 0.50, 0.75 and 1 mM) was mixed with 225 μL of first reagent (Acyl-CoA synthetase (ACS), Coenzyme A (CoA) and ATP) and incubated 3 min at 37°C and absorbance was measured at 595 nm (sample blank measurement), followed by addition of 75 μL of the second reagent containing Acyl-CoA oxidase

(ACOD), peroxidase (POD), Methyl-Ethyl-Hydroxymethyl-Alanine (MEHA); the plate was incubated for another 15 min and final absorbance read at 595 nm.

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 were made to include the trailing edge of the filament where the chloride cells reside. Gill sections were immune stained according to Dang and colleagues (Dang, Lock, Flik & Wendelaar Bonga 2000) as described in detail for African catfish (Schram *et al.* 2010).

Blood haematocrit levels

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

Specific growth rate, feed intake and feed conversion ratio

On day 0 and day 42, the fish in each tank were individually weighed (Mettler PM 34 Delta range, Mettler-Toledo BV, Tiel, The Netherlands) to the nearest 1 g, to calculate the specific growth rate (SGR) as follows:

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

Where SGR = specific growth rate (% d^{-1}), W_t = mean weight at day 42 (g), W_0 = mean weight at day 0 (g) and t = number of days.

Floating feed (Catfish type Me-3; Skretting, Boxmeer, the Netherlands) with 49% crude protein and 11% crude lipids was given twice daily at 9 am and 3 pm until apparent satiation (no more feed taken for at least 5 min following administration of the feed). Feed loads per tank were recorded daily. All uneaten pellets were collected from each tank 1 h after each of the two daily feeding sessions. Feed loss per tank was calculated as the total number of uneaten feed pellets multiplied by 0.0966 g $pellet^{-1}$, the average weight of a pellet, determined by weighing 100 feed pellets. Daily feed intake per tank resulted from the difference between daily feed load and daily feed loss. Daily feed intake per tank was divided by the number of fish in the tank to calculate the daily feed intake per fish in each tank. For each tank the total feed intake per fish (TFI) was determined by summation of daily feed intake per fish in each tank. Total feed intake per fish and biomass increase per fish were used to calculate feed conversion ratio (FCR) as follows:

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

Where FCR = feed conversion ratio (g g^{-1}), TFI = total feed intake (g $fish^{-1}$), W_t = mean individual weight at day 42 (g) and W_1 = mean individual weight at day 0 (g).

Statistics

Physiological parameters

Physiological parameters are expressed as mean (SD) of the individual measurements per treatment. For each treatment, 26 fish had been sampled; in some instances not all samples were analysed because of insufficient plasma volume. When necessary, data were log transformed to obtain residuals that were approximately 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 tank as a random effect (F-tests with Kenward-Roger approximation to the residual degrees of freedom (Kenward & Roger 1997)). Statistical analyses were performed in SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Only when significant treatment effects were detected, a least significance 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%.

Plasma nitrate concentrations and plasma nitrate to water nitrate ratios were related to water nitrate concentrations by linear regression analyses with water nitrate concentration as fixed effect and tank as a random effect. Plasma nitrite concentrations were related to water nitrite and plasma nitrate concentrations by linear regression

analyses with either water nitrite or plasma nitrate concentration as fixed effect and tank as a random effect. Plasma chloride concentrations were related to plasma nitrate concentrations by linear regression analysis with plasma nitrate concentration as fixed effect and tank as a random effect. In all regression analyses F-tests with Kenward-Roger approximation to the residual degrees of freedom were used (Kenward & Roger 1997). The pair-fed and sodium chloride groups were not considered in regression analyses.

Feed intake and growth

Initial and final individual weight, total feed intake per fish (TFI), 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 in SAS 9.2 (SAS Institute Inc., Cary, North Carolina, USA). Only in case significant treatment effects were detected, a least significance difference (LSD) post hoc analysis was used to estimate the level of significance between mean values. For both ANOVA and LSD analysis the fiducial limit was set at 5%.

Concentration-effect curves and NOEC

Nitrate concentration-effect curves were fitted for specific growth rate (SGR) and total feed intake per fish (TFI) using a log-logistic model (Seefeldt, Jensen & Fuerst 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., San Diego, CA, USA). The 10% effect concentrations (EC_{10}) and their 95% confidence limits were calculated (Miller & Miller 2000). No observed effect concentrations (NOEC) were determined for SGR and TFI as the highest nitrate concentrations in the experiment at which no significant difference with the control treatment were observed.

Results

Plasma nitrate and nitrite concentrations

Nitrate concentrations in the water had a strong effect on plasma nitrate (NO_3^-) concentrations. Plasma nitrate concentrations were significantly

Table 2 Mean (SD) values at the start ($t = 0$) and per treatment for the end ($t = 42$ days) of the experiment for plasma NO_3^-, plasma NO_3^- to water NO_3^- ratio, plasma NO_2^-, plasma Cl^-, branchial Na^+/K^+-ATPase activity and plasma osmolality. 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

	Water NO_3	Plasma NO_3		Plasma NO_3/Water NO_3		Plasma NO_2		Plasma Cl^-		Na^+/K^+-ATPase activity		Plasma osmolality	
Treatment	**(mM)**	**(μM)**	***n***		***n***	**(μM)**	***n***	**(mM)**	***n***	**(μmol Pi h^{-1} mg $protein^{-1}$)**	***n***	**(mOsmol kg^{-1})**	***n***
1-$t = 0$		59 (14)	10			3.4 (1.0)	9	111.6 (12.8)	26	1.2 (0.4)	10	268.6 (5.5)	25
2-control	0.39	71 (29)^{a}	10	0.18 (0.07)	10	3.4 (2.8)^{ac}	10	108.7 (6.8)^{a}	26	1.4 (0.7)	9	269.3 (6.4)	26
3	1.46	282 (99)^{b}	10	0.19 (0.07)	10	2.3 (1.2)^{ab}	10	108.9 (11.5)^{a}	26	0.9 (0.5)	10	271.5 (6.6)	25
4	4.21	623 (202)^{c}	10	0.15 (0.05)	10	1.2 (0.5)^{b}	9	102.6 (7.6)^{a}	26	1.2 (0.5)	10	269.2 (6.3)	26
5	9.69	2076 (872)^{d}	12	0.21 (0.08)	12	4.9 (3.8)^{ac}	11	101.0 (9.8)^{ac}	26	1.0 (0.5)	9	267.6 (4.8)	26
6	27.04	6623 (921)^{e}	13	0.25 (0.05)	13	7.9 (9.0)^{c}	17	86.5 (9.7)^{b}	22	1.7 (1.0)	10	264.9 (7.2)	23
7-pair-fed	0.40	85 (26)^{a}	10	0.21 (0.06)	10	4.5 (2.3)^{ac}	10	113.5 (9.4)^{a}	23	1.2 (0.5)	9	267.6 (5.0)	23
8-chloride	22.49	7011 (996)^{e}	14	0.31 (0.03)	14	5.1 (4.3)^{ac}	14	89.1 (11.1)^{bc}	24	1.0 (0.7)	10	269.1 (5.7)	26
P-value		<0.0001		0.20		0.049		0.01		0.70		0.47	

different among all treatments (Table 2) and found to increase linearly with increasing nitrate concentrations in the water (Table 3). Pair feeding nor (sodium) chloride addition to the water affected plasma nitrate levels (Table 2). Plasma nitrate to water nitrate ratios ranged from 0.15 to 0.25 among the nitrate treatments. The highest value (0.31) was observed in the high nitrate exposure level (22.5 mM) combined with sodium chloride. However, treatments effects on the plasma to water nitrate ratios were not detected (Table 2 and 3). Plasma nitrite (NO_2^-) differed among treatments (Table 2) but at 8 μM the highest value observed is still low and only slightly higher than control values (3.4 μM). The increase in plasma nitrite could not be attributed to differences in water nitrite concentrations (Table 3). Instead, the plasma nitrite concentration was found to increase linearly with increasing water nitrate and plasma nitrate concentrations (Table 3).

Plasma chloride and plasma osmolality

Significantly different plasma chloride concentrations were detected among treatments (Table 2). Plasma chloride concentrations were found to decrease linearly with increasing plasma nitrate concentration (Table 3). Plasma osmolality was unaffected by the treatments (Table 2).

Haematocrit, plasma cortisol, glucose, lactate and NEFA, and branchial Na+/K+-ATPase activity

As shown in Table 4, no significant differences in plasma concentrations of cortisol, glucose, lactate and NEFA, branchial Na^+/K^+-ATPase activity and haematocrit were observed. All values were within normal ranges previously reported for African catfish, *Clarias gariepinus* (Schram *et al.* 2010).

Gill morphology

Gill morphology, presented for controls (0.39 mM NO_3^-), pair fed (0.40 mM NO_3^-), highest (27.0 mM NO_3^-) and chloride (22.5 mM NO_3^-), was not affected by nitrate exposure (Fig. 1). The number of chloride cells, nor their location within the gills were affected (data not shown).

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

Four fish died during the experiment out of a total of 208 fish. Death did not result in significantly different survival rates among treatments (Table 5). Total feed intake per fish differed among treatments (Table 5). At the highest nitrate level (27.0 mM) and the high nitrate exposure level (22.5 mM) combined with sodium chloride, the total feed intake per fish was reduced by over 50% compared to the groups exposed to nitrate levels of 9.7 mM or lower. The differences in feed intake among treatments became apparent already 1 day after the highest nitrate exposure concentration had been reached (Fig. 2).

Specific growth rate (SGR) differed among treatments (Table 5). At the highest nitrate exposure level (27.0 mM), in the pair-fed group and at the high nitrate exposure level (22.5 mM) combined with sodium chloride, SGR was significantly reduced by at least 35% compared to the groups exposed to nitrate levels of 9.7 mM and lower.

The highest value for feed conversion ratio was seen in the 22.5 mM-NO_3^- with sodium chloride group (0.92); the FCR of 0.84 at the highest

Table 3 Results of linear regression analyses with tanks as random effect

		Regression coefficient		Intercept	
Response variable	**Explanatory variable**	**Estimate**	***P*-value***	**Estimate**	***P*-value**
Plasma nitrate (mM)	Water nitrate (mM)	0.2442	<0.0001	−0.1857	0.52
Plasma chloride (mM)	Plasma nitrate (mM)	−3.601	0.0005	108.06	<0.0001
Plasma nitrate to water nitrate ratio	Water nitrate	0.0026	0.14	0.171	<0.0001
Plasma nitrite (μM)	Water nitrite (μM)	0.152	0.56	2.51	0.25
Plasma nitrite (μM)	Plasma nitrate (μM)	0.000624	0.0075	2.42	0.0020
Plasma nitrite (μM)	Water nitrate (μM)	0.000130	0.026	2.48	0.0037

*Equals model *P*-value.

Table 4 Mean (SD) values at the start (t = 0) and per treatment for the end (t = 42 days) of the experiment for plasma cortisol, plasma glucose, plasma lactate and NEFA 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

	Water NO_3	Plasma cortisol		Plasma glucose		Plasma lactate		Plasma NEFA		Haematocrit	
Treatment	**(mM)**	**(nM)**	***n***	**(mM)**	***n***	**(mM)**	***n***	**(mM)**	***n***	**(%)**	***n***
1-t = 0		15.2 (25.2)	22	3.28 (1.1)	26	4.79 (1.0)	26	0.17 (0.07)	21		
2-control	0.39	9.6 (6.2)	22	3.54 (1.0)	26	4.97 (1.2)	26	0.17 (0.11)	18	32.1 (4.3)	24
3	1.46	14.2 (9.8)	24	3.72 (0.9)	26	5.02 (1.1)	26	0.22 (0.11)	18	29.0 (5.1)	26
4	4.21	20.4 (16.3)	25	2.89 (0.9)	26	4.84 (0.9)	26	0.23 (0.14)	20	27.0 (3.9)	25
5	9.69	10.8 (12.8)	23	3.00 (0.4)	26	4.96 (0.9)	26	0.15 (0.07)	19	28.8 (5.3)	26
6	27.04	19.5 (16.7)	23	2.92 (0.5)	23	5.51 (1.5)	23	0.22 (0.13)	16	32.4 (5.4)	23
7-pair-fed	0.40	17.6 (11.0)	23	3.36 (0.8)	20	4.10 (0.7)	23	0.23 (0.12)	22	25.7 (4.8)	22
8-chloride	22.49	20.3 (12.7)	25	2.58 (0.4)	25	5.61 (1.2)	25	0.24 (0.11)	25	31.7 (4.5)	23
P-value		0.71		0.43		0.30		0.54		0.08	

nitrate exposure level (27.0 mM) was not different from the values calculated for pair-fed controls.

EC_{10} and NOEC for total feed intake and SGR

The concentration-effect curves for TFI and SGR in relation to the water NO_3 concentration (Figs. 3a and 3b), yield and EC_{10} for NO_3^- of 22 mM (312 mg L^{-1} NO_3^--N), with a 95% confidence interval from 20 to 25 mM when read against TFI. For SGR, a very similar EC_{10} for NO_3^- of 23 mM (321 mg.L^{-1} NO_3^--N), with a 95% confidence interval from 21 to 26 mM was calculated. For both SGR and TFI the highest test concentration at which no significant difference with the lowest nitrate exposure concentration was observed (NOEC) was 9.7 mM.

Discussion

Plasma nitrate and nitrite concentrations

In *Clarias gariepinus*, plasma nitrate concentrations increase with increasing nitrate concentrations in the water. Nitrate transport mechanisms have not been established in fish to date. It has been suggested that nitrate is only passively moving across the gills of rainbow trout, *Oncorhynchus mykiss* (Stormer *et al.* 1996). In that study on trout, an ambient nitrate concentration of 1 mM resulted in a five times lower plasma concentration (0.2 mM). We observed very similar ambient to plasma nitrate ratio in our African catfish study, suggesting similar nitrate handling in both species. These low molar ratios between plasma nitrate and ambient nitrate suggest that the integument forms a significant barrier to waterborne nitrate.

Nitrate has, *in vitro*, low, millimolar, affinity for branchial chloride carriers (Stormer *et al.* 1996). This is confirmed *in vivo* in our study as addition of (sodium) chloride to the water (next to high nitrate) did not affect plasma nitrate levels nor nitrate toxicity in African catfish.

As branchial nitrate permeability appears to be low, other nitrate uptake routes also need to be considered. Gastro-intestinal nitrate exposure via water ingestion may become significant when the fish starts drinking or ingests water during feeding. Intestinal uptake of nitrate has not yet been examined in fish but for nitrite this pathway is well documented in European flounder, *Platichthys flesus* (Grosell & Jensen 2000). Intestinal uptake

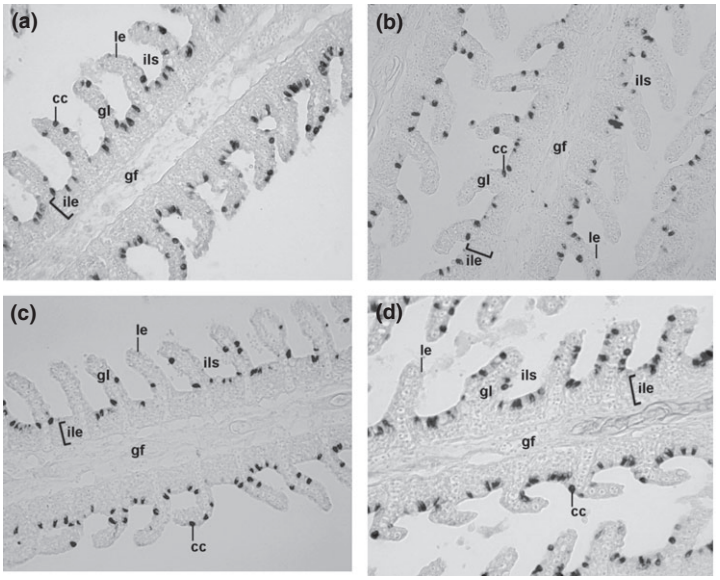

Figure 1 Histology of gill epithelium immunohistochemically stained for Na^+/K^+ ATPase-rich cells (chloride cells) of the control (3a), pair fed (3b), 27.0 mM NO_3 (3c) and 22.5 mM NO_3 in addition with NaCl (3d) treatment groups. No effects on the gill's morphology with increasing water nitrate level were observed. Legend: ile = inter-lamellar epithelium, le = lamellar epithelium, ils = inter-lamellar space, cc = chloride cell, gf = gill filament, gl = gill lamellae.

Table 5 Mean (SD) values per treatment ($N = 2$) for initial weight, final weight, total feed intake (TFI), specific growth rate (SGR), feed conversion ratio (FCR) and survival rate. Mean values with different superscripts are significantly different (one-way ANOVA, P values as shown). SD = Standard deviation of mean values per treatment

Treatment	Water NO_3 (mM)	Initial weight (g)	Final weight (g)	TFI (g $fish^{-1}$)	SGR (%BW d^{-1})	FCR	Survival (%)
1-t = 0		152.6 (3.1)					
2-Control	0.39	153.6 (9.0)	495.5 (5.6)^a	262.4 (0.6)^a	2.93 (0.18)^a	0.77 (0.03)^a	100 (0)
3	1.46	155.1 (12.4)	480.3 (12.8)^a	251.4 (4.4)^a	2.83 (0.13)^a	0.77 (0.01)^{ab}	100 (0)
4	4.21	157.2 (5.7)	483.3 (28.6)^a	252.9 (17.9)^a	2.81 (0.06)^a	0.78 (0.00)^{ab}	100 (0)
5	9.69	151.6 (7.2)	464.7 (48.9)^a	246.9 (23.6)^a	2.80 (0.14)^a	0.79 (0.03)^{ab}	100 (0)
6	27.04	149.4 (9.6)	303.5 (91.7)^b	127.3 (60.8)^b	1.72 (0.60)^b	0.84 (0.05)^b	88 (16)
7-Pair-fed	0.40	155.0 (5.4)	330.9 (16.5)^b	136.0 (12.9)^b	1.90 (0.21)^b	0.77 (0.02)^{ab}	88 (16)
8-Chloride	22.49	158.3 (13.4)	278.9 (31.6)^b	111.4 (19.5)^b	1.41 (0.07)^b	0.92 (0.02)^c	100 (0)
P-value		0.96	0.004	0.002	0.002	0.009	0.58

may explain why turbot, a marine and therefore drinking species, is relatively sensitive to waterborne nitrate (Van Bussel, Schroeder, Wuertz & Schulz 2012). From our studies we cannot discriminate a contribution of ingested nitrate to nitrate accumulation in the plasma but the reduced feed intake we observed in our fish exposed to high nitrate may be a behavioural adaptation aimed at reducing nitrate exposure via ingested water. Fish moisten dry feed pellets with water and this may contribute to intake of nitrate. Increased ionic levels in fresh water could further induce drinking (Hirano 1974). It should be noted that the addition of the highest concentration of nitrate in this experiment was less than 30 mM $NaNO_3$, equivalent to an increase in water osmolality of about 60 mOsmol kg^{-1}. In media of such low osmolality passive/diffusional water influx may be significant and therefore drinking an unwanted behaviour. Independently of what the route of entry is, apparently millimolar plasma concentrations of nitrate are tolerated by African catfish. This is in accordance with the consensus on the robustness of this fish in aquaculture.

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

High levels of nitrate in the water did not affect plasma osmolality in this catfish. As Cl^- (with Na^+) is a major determinant of plasma osmolarity, the hypochloraemia (86.5 (9.7) mM versus 108.7 (6.8) mM in controls) observed at the highest

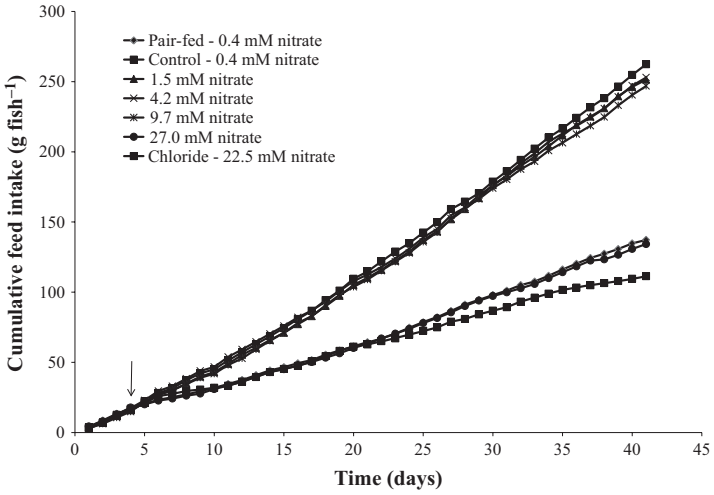

Figure 2 Mean ($N = 2$) cumulative feed intake per fish during the experimental nitrate exposure. The arrow indicates the first day at which all treatments reached their designated nitrate concentrations.

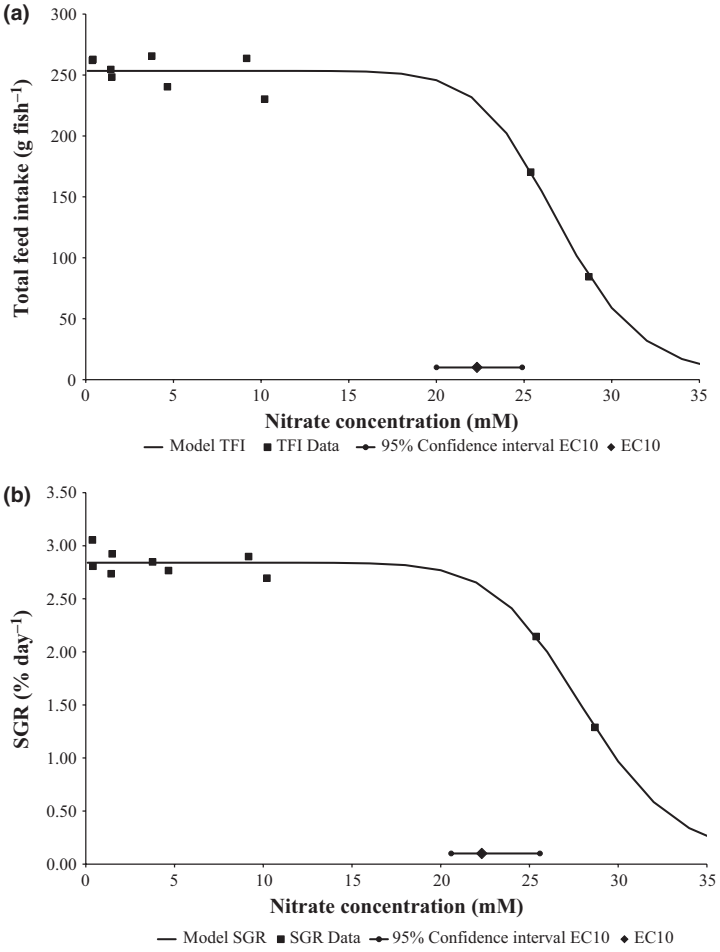

Figure 3 Concentration-effect curves for total feed intake (TFI) (a) and specific growth rate (SGR) (b) in relation to the water NO_3 concentration($[NO_3]$, mM). TFI = $253.4–253.4/(1 + 10^{((1.432-\log[NO3])/0.0869)})$ and SGR = $2.84–2.84/(1 + 10^{((1.450-\log[NO3])/0.0936)})$. Pair-fed and chloride treatments were not included.

ambient nitrate levels could have induced a 20 mOsmol kg^{-1} decrease in osmolality of the plasma compartment. The high plasma nitrate levels (>6 mM) may have compensated, at least partly, hypochloraemic effects on plasma osmolality. Whereas nitrate transport is well known in

plants and yeast (Orsel, Filleur, Fraisier & Daniel-Vedele 2002; Machín, Medina, Navarro, Pérez, Veenhuis, Tejera, Lorenzo, Lancha & Siverio 2004), to date, no literature seems to exist on nitrate transport proper in animal tissues. Nitrate may interfere and reduce chloride reabsorption in the distal mammalian nephron (Kahn, Bosch, Levitt & Goldstein 1975), but specific nitrate transporter mechanisms have, to the best of our knowledge, not been demonstrated in animal osmoregulatory organs. The hypochloraemia seen at the highest ambient nitrate levels and the coinciding millimolar plasma nitrate levels do suggest that plasma nitrate interferes with chloride handling. In a freshwater fish, branchial and intestinal chloride uptake from water and food and renal reabsorption are key to chloride homeostasis (Fuentes, Soengas, Rey & Rebolledo 1997) and these are the sites where nitrate could interfere to explain the hypochloraemia.

Nitrate exposure did not affect branchial Na^+/K^+-ATPase activity. The enzymatic activities measured in this experiment are somewhat lower than the values reported recently for the same species (Schram *et al.* 2010), which we attribute to salinity differences between the current (0.4–2.4 g L^{-1}) and the previous ammonia study (4 g L^{-1}). Apparently a significant hypernitrataemia 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 nitrate exposure. Nitrate exposure also did not cause morphological changes nor anomalies of the branchial epithelium. Morphological adaptations to reduce the permeability of the gills were observed when this fish species was exposed to increasing ambient ammonia concentrations (Schram *et al.* 2010). The results jointly taken indicate that high levels of nitrate do not affect permeability of the gills, neither to water or ionic species central to osmotic homeostasis, nor to nitrate itself (Stormer *et al.* 1996) as we conclude from the insignificant increase in molar ratios for nitrate in water and plasma; clearly, nitrate is not very toxic for this fish species.

Stress physiology

None of the stress and energy metabolite parameters (plasma cortisol, glucose, lactate and NEFA) were affected by nitrate. All parameters showed values within the range considered normal and reported before for the same species (Schram *et al.* 2010). Even the highest nitrate exposure concentration apparently did not impose distress.

Feeding

During the first 4 days of the experiment when nitrate concentrations were building up, feed intake was similar among all treatments. On day 5, however, when maximal nitrate levels had been reached, significant differences in feed intake showed up more or less instantaneously at the highest nitrate levels (22.5 and 27.0 mM). Feed intake instantly drops above 10 mM nitrate in the water; apparently a threshold is surpassed where appetite is lost. We conclude from this that nitrate is apparently 'sensed' by this fish. The effect of high ambient nitrate on feed intake appears direct and persistent, as feed intake dropped within 24 h and remained low for up to 42 days.

A pair-fed group was introduced to discriminate effects of nitrate exposure from potential effects of reduced feed intake. No significant differences were observed in physiological parameters for any treatment. However, feed intake, feed conversion ratio and specific growth rate were equal for catfish exposed to the highest nitrate level and their pair-fed controls. We conclude from this observation that high nitrate exposure must inflict upon appetite or feeding behaviour. The highest feed conversion ratio (0.92) seen in the group exposed to high nitrate concentration (22.5 mM) together with sodium chloride suggests that in these fish osmoregulatory activity imposes an extra energy demand.

Nitrate toxicity

Camargo *et al.* (2005) suggested that conversion of haemoglobin to methaemoglobin and loss of oxygen carrying capacity is at the basis of nitrate toxicity, similar to the well-documented mechanism of nitrite toxicity (Eddy & Williams 1987; Williams *et al.* 1993). Direct evidence for this mode of action for nitrate toxicity in fish is limited to one study on rainbow trout (Grabda, Einszporn-Orecka, Felinska & Zbanysek 1974) and, for a similar action on haemocyanin in a penaeid shrimp *Marsupenaeus japonicus* (Cheng & Chen 2002). In contrast, elevated methaemoglobin was not observed in nitrate-exposed rainbow trout in a study by Stormer *et al.* (1996). According to Bodansky (1951), nitrate is first reduced to nitrite before oxidizing haemoglobine to methaemoglobin. Unfortunately, we failed to

determine methaemoglobin in our nitrate-exposed catfish. However, the findings of Stormer *et al.* (1996), Bodansky (1951) together with the plasma nitrate and haematocrit data from our study do suggest although that other mechanisms for nitrate toxicity than direct haemoglobin oxidation need to be considered. Plasma nitrate levels of 7 mM did not cause severe adverse effects in African catfish, while very similar plasma nitrite levels were reported to be lethal in fish (5–9 mM in channel catfish, *Ictalurus punctatus* (Lewis & Morris 1986), 8 mM in salmonids (Eddy *et al.* 1983)).We conclude from this that it is unlikely that nitrate significantly converted haemoglobin to methaemoglobin. This notion is supported by our observation that nitrate exposure did not affect haematocrit levels. Decreased haematocrit was observed in nitrite exposed rainbow trout (Stormer *et al.* 1996) and common carp (*Cyprinus carpio)* (Jensen 1990), an effect attributed to increased red blood cell turnover, due to nitrite-induced methaemoglobinaemia.

The slightly elevated plasma nitrite we observed in African catfish was related to plasma nitrate rather than water nitrite. Elevated plasma nitrite could originate from plasma nitrate when cells reduce nitrate to nitrite (Lundberg, Weitzberg & Gladwin 2008) which then appears in the plasma compartment, but mechanisms for such route have not been documented in fish to date (Sandvik, Nilsson & Jensen 2011). Our results indicate that nitrate–nitrite conversion could be present in African catfish. It is, however, not likely that nitrite-induced methaemoglobinia is at the basis of treatment effects rather than the nitrate exposure. The presence of methaemoglobin reductase inside the red blood cells (Cameron 1971) would keep pace with any slightly elevated haemoglobin oxidation rate caused by this slightly elevated plasma nitrite, maintaining methaemoglobin low.

Nitrate threshold concentrations

African catfish chronically exposed to 27.0 mM ambient nitrate did not show major physiological disturbances. The threshold concentration for chronic nitrate exposure of African catfish is in our view therefore best based on nitrate effects on feed intake and growth. As only the two highest tested concentrations (22.5 and 27.0 mM) induced a significant effect, we propose to use the highest test concentration that showed no significant effect (no observed effect concentration, NOEC) as a safe threshold concentration for nitrate (9.7 mM, 140 mg L^{-1} NO_3-N) and not the calculated EC_{10}.

Conclusions

Nitrate may be considered an end product and stable non-toxic form of nitrogenous waste, and this notion fits in with millimolar plasma levels of nitrate. However, ambient levels above 10 mM nitrate affected food intake and reduced growth, and rather instantly. We therefore advise for African catfish, *Clarias gariepinus* not to exceed a water nitrate concentration of 10 mM (140 mg L^{-1} NO_3-N). Below this nitrate concentration physiological and growth disturbances are avoided. We further advise to avoid incidental high nitrate as African catfish may immediately stop feed intake. Mildly elevated salinity (by sodium chloride addition) does not reduce effects of high nitrate exposure in African catfish.

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