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Chronic exposure to nitrate significantly reduces growth and affects the health status of juvenile Nile tilapia (*Oreochromis niloticus* L.) in recirculating aquaculture systems

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Key words: feed conversion; specific growth rate; methemoglobin, gill histology, nitrate; nitrite

Abstract

Studies on chronic or acute toxicity of nitrogen species on fish in recirculating aquaculture systems (RAS) usually focused on adverse effects of total ammonia nitrogen (TAN: sum of NH_3 + NH_4^+) and nitrite (NO_2^-), while underestimating the potential effects of high nitrate accumulation on growth and health status of fish. In our study, Nile tilapia (*Oreochromis niloticus*) were exposed to five different nitrate concentrations (0, 10, 100, 500 and 1000 mg L^{-1} NO_3^--N) over 30 days. Growth parameters (feed conversion ratio: FCR, specific growth rate: SGR, hepatosomatic index: HSI), blood samples (concentrations of hemoglobin, methemoglobin, plasma NO_2^-/NO_3^-) and the histology of the gills were studied to evaluate growth and health status of the fish. At the highest nitrate concentration, the fish showed significantly reduced growth and impaired health status (SGR, FCR, plasma NO_2^-/NO_3^-, hemoglobin- and methemoglobin concentration), demonstrating that too high nitrate concentrations can negatively influence tilapia production in RAS. Here, we recommend not exceeding concentrations of 500 mg L^{-1} NO_3^--N in juvenile tilapia culture to ensure an optimal health and growth status of the fish, since below that concentration no effects on the tilapia have been observed.

Introduction

Recirculating aquaculture systems (RAS) have been rapidly evolving over the last two decades and are envisioned a great potential with regard to a sustainable aquaculture development due to the efficient use of water and space as well as minor environmental impact (Gutierrez-Wing & Malone 2006). However, a major drawback of RAS is the accumulation of waste products such as nitrate after biofiltration. As a consequence of improved recirculation technology and subsequently decreasing water exchange, waste products such as nutrients are accumulating in the process water (van Rijn 2013). Compared to open aquaculture systems like ponds, net cages

or semi-closed systems where these products are of minor relevance to the cultured species due to high water exchange, concentrations may exceed critical levels impacting welfare as well as performance of the fish. This is particularly relevant for aquaponics, where high nitrate concentrations originating from a RAS-based fish production are desirable to fertilize the plants in the hydroponic unit. Here, nitrate concentrations in the range of 150 - 230 mg L^{-1} NO_3^--N are recommended e.g. for the hydroponic production of tomatoes, cucumbers and peppers (Lattauschke 2004)

Biofiltration in RAS is necessary to convert toxic total ammonia nitrogen (TAN) via nitrite to nitrate (Timmons, Holder & Ebeling 2006). Based on the experience in open systems and the respective concentrations, nitrate has been considered harmless to the fish (Rakocy, Masser & Losordo 2006) and only limited attention was directed to the adverse effects of nitrate in the past. However, in contrast to ponds and other open systems, nitrate can accumulate to concentrations of up to 1000 mg L^{-1} NO_3^--N in RAS (van Rijn 2010). Therefore, potential chronic effects on growth and health of fish become more likely. Furthermore, problems interfering with the production efficiency may emerge due to reduced growth performance caused by high nitrate concentrations.

The conversion of hemoglobin to methemoglobin has been reported as the main mechanism of nitrate toxicity on aquatic animals (Jensen 1996; Scott & Crunkilton 2000; Cheng & Chen 2002), but alternative modes of action (MOA) have been discussed including pathological impairment of the gills, immune suppression and endocrine effects on the thyroid system as well as on androgens and estrogens (Camargo, Alonso & Salamanca 2006; Davidson, Good, Welsh & Summerfelt 2014; Hamlin, Moore, Edwards, Larkin, Boggs, High, Main & Guillette 2008, Freitag, Thayer, Leonetti, Stapleton & Hamlin 2015). In a 30 day trial, nitrate modulated the conversion of steroids at 57 mg L^{-1} NO_3^--N, affecting key players – testosterone, 11-

74 ketotestosterone and estradiol - in the endocrine regulation of growth and reproduction (Hamlin
75 et al. 2008) and concentrations as low as 10 mg L^{-1} NO_3^--N raised testosterone in Atlantic salmon
76 (Freitag et al. 2015). In mosquitofish, embryonal dry weight was reduced and reproductive
77 behavior of mature females was affected at minimal concentrations of 5 mg L^{-1} NO_3^--N
78 (Edwards, Miller & Guillette 2006). Moreover, elevated nitrate concentrations up to 110 mg L^{-1}
79 NO_3^--N lead to a decrease in the thyroid hormones T3 and T4 in rats (Eskiocak, Dundar, Basoglu
80 & Altaner 2005). Impact on swimming performance and survival in juvenile rainbow trout has
81 already been reported at 91 mg L^{-1} NO_3^--N (Davidson et al. 2014). Still, substantially reduced
82 growth performance might be the most relevant for the farmer in terms of economic impact. At
83 increasing nitrate concentrations, linear decrease in specific growth rate (SGR) was observed in
84 turbot (*Scophthalmus maximus*) resulting in a dramatically reduced SGR (30 %) at 500 mg L^{-1}
85 NO_3^--N (van Bussel, Schroeder, Wuertz & Schulz 2012). Similarly, Schram, Roques, Abbink,
86 Yokohama, Spanings, de Vries, Bierman, van de Vis & Flik (2014, a) observed reduced growth
87 performance in African catfish (*Clarias gariepinus*) at nitrate concentrations >140 mg L^{-1} NO_3^--
88 N. Consequently, adverse effects need to be evaluated for one of the most important species in
89 intensive aquaculture, where concentrations above 100 mg L^{-1} NO_3^--N are regularly observed and
90 thus may be relevant upon chronic exposure.
91 In contrast, acute toxicity of nitrate in fish is often observed at extreme concentrations, where
92 96 h LC50 were observed between 1,250 mg L^{-1} NO_3^--N and 1,400 mg L^{-1} NO_3^--N e.g. in
93 rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*) and Chinook salmon
94 (*Oncorhynchus tshawytscha*) in separate studies (Tomasso & Carmichael 1986; Colt &
95 Tchobanoglous 1976; Westin 1974). Despite the importance of tilapia aquaculture globally (FAO
96 2012), no data on chronic effects of nitrate exposure and safe threshold concentrations have been
97 published so far. In addition, the uptake of nitrate in fish is not yet comprehensively described,

but essential to understand nitrate toxicity in fish. Compared to NH_3 or NO_2^- nitrate uptake is presumably low as a result of low branchial permeability towards nitrate (Stormer, Jensen & Rankin 1996). Still, relatively high plasma concentrations of NO_x (sum of NO_2^- and NO_3^-) have been reported upon nitrate exposure (Schram et al, 2014 a,b; Stormer et al., 1996). Consequently, alternative uptake routes and sites may be involved.

The objective of the present study was to identify potential effects of high nitrate concentrations on growth and health status of juvenile Nile tilapia. Therefore an exposure experiment was conducted with juvenile Nile tilapia to assess the impact of nitrate in intensive aquaculture. Based on the results we give a recommendation for safe levels of nitrate in the production of juvenile Nile tilapia. In a second experiment, the reduction of nitrate to nitrite in the stomach juice was studied *in vitro* over time to clarify if nitrate conversion and subsequent nitrite uptake is an alternative uptake route to direct uptake of nitrate, considering the plasma concentrations of nitrite and nitrate observed *in vivo*.

Material and Methods

Experimental setup

We conducted an experimental NO_3^- exposure of juvenile tilapia (total length 8.8 ± 0.48 cm, wet weight 13.5 ± 2.5 g) at concentrations of 0, 10, 100, 500 and 1000 mg L^{-1} NO_3-N (0, 0.7, 7, 36, 70 mM) over a 30 d period in a continuous flow-through system. Tilapia were individually stocked to forty 9 L glass aquaria (30×20×14.5 cm) with an overflow providing 7 L of rearing volume (flow rate 50 L/d). All aquaria were placed in a water bath and aerated, assuring a constant temperature of 27.3°± 0.3°C (min 26.0 °C, max 28.9°C) and 7.8 ± 0.3 mg/L O_2 (100 % O_2). Fish were fed a commercial food (Aller Futura Ex, Emsland-Aller Aqua, Germany) at 1.5 % of their body weight per day.

After acclimatization for one week, respective concentrations were established by flow controlled assembly consisting of a peristaltic pump, a rotameter flow gauge, a needle valve and a mixing chamber, diluting a 100fold stock solution with prefiltered, temperature conditioned tap water (Lutz, Kloas, Springer, Holden, Wolf, Krueger, & Hosmer 2008). The stock solution was formulated with $NaNO_3$ and KNO_3 at Na^+/K^+ weight ratio of 6.2 : 1 considering the mean ratio in the Nile (Zimmermann-Timm 2011; Dekov, Komy, Araujo, Van Put & Van Grieken 1997; Komy & El-Samahy 1995) to avoid disturbances in cellular homeostasis (van Bussel et al. 2012). $NaNO_3$ and KNO_3 were food quality grade (CHEM-DIS, Eisenberg, Germany). Each mixing chamber supplied four aquaria, referred to as cluster. For each treatment, there where two clusters assessing eight fish in total. Flow rates of nitrate stock solutions were controlled and adjusted twice a day, flow rates of tap water were controlled on a weekly basis. Temperature, pH and oxygen concentration were determined daily with a portable multimeter (HQ40d multi, Hach Lange GmbH, Germany). Salinity was measured three times over the experimental period with a portable meter (WTW LF92, WTW GmbH, Weilheim, Germany). The experiment was conducted in compliance with the local animal welfare committee (LAGESO G0367/12).

Concentrations (mg L^{-1}-N) of TAN, NO_2^- and NO_3^- in the water were determined every second day by the cadmium reduction method, the diazotization method and the ammonia salicylate method using a spectrophotometer DR3900 (Hach Lange GmbH, Germany).

Sampling

After 30 days, fish were killed and blood samples were taken from the caudal vein with heparinized syringes. Samples for the determination of hemoglobin were kept on ice and analyzed within 3 h. For methemoglobin, whole blood samples were shock frozen and stored at –80°C. Blood plasma was obtained by centrifugation (5000 *g*, 2 min), shock frozen and stored at –80°C. Fish were weighed to the nearest 0.1 g and length was recorded to the nearest of 1 mm,

liver to the nearest of 1 mg. The HSI was calculated as HSI = (liver weight / final weight of fish) *100. For histology, the fourth right gill arch was dissected and fixed in 10 % phosphate buffered formaldehyde solution (Histofix, Carl Roth, Germany).

Plasma concentrations of NO_2^- and NO_3^-

We measured the sum of nitrite and nitrate (NO_x) as well as nitrite in the plasma using the nitrate/nitrite colorimetric assay kit (Cayman, USA) according to the user's manual. Briefly, for NO_x and NO_2^- determination, plasma was diluted 1:20 prior measurement. Absorbtion was determined at 530 nm with an Infinite M200 microplate reader (Tecan Trading AG, Switzerland). All samples were analyzed in duplicate. The NO_3^- concentration was then calculated as NO_x – NO_2.

Hemoglobin and methemoglobin determination

Total hemoglobin was determined within 3 h upon sampling with a diagnostic hemoglobin kit (DiaSys Diagnostic Systems, Germany) and calculated from a standard dilution series (12 g/dL hemoglobin standard, HEM QS, Diaglobal, Germany) as described in Wuertz, Schulze, Eberhardt, Schulz & Schroeder (2013). For the methemoglobin concentration the ratio of Meth-Hb and total-Hb was determined using the cyan ferrocyancomplex method according to Hegesh, Gruener, Cohen, Bochkovsky & Shuval (1970). Briefly, 20 µL blood was incubated (15 min) in 1 mL pure water. After addition of 600 µL saponin solution (1% saponin, 14 mM Na_2HPO_4, 42 mM KH_2PO_4, pH 6.6) and vortexing, cell debris were separated by centrifugation (10 min, 3000 *g*). Samples were analyzed in duplicates, measuring the absorption at 633 nm in (A1) 250 µL supernatant, (A2) after the addition of 5 µL 1% KCN and incubation for 10 min, in (A3) 250 µL supernatant after addition of 5 µL $K_4[Fe(CN)_6]$, followed by an addition of 5 µL 1% KCN and incubation for 10 min (A4). Total Hb:MetHb was calculated as (A1–A2)/(A3–A4).

Gill histology

After fixation in phosphate-buffered formalin for approximately 24 h at 4°C, samples were transferred to embedding cassettes and washed three times with 0.1 M phosphate buffer [0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, pH 7.3]. The last washing step was carried out overnight. Samples were dehydrated with successive washes of EtOH (70 %, 96 %, 100 %, 100 %) for 1 h each. Preinfiltratation was carried out with a 1:1 ethanol Technovit 7100 solution for 1 h, followed by infiltration in 100 mL Technovit 7100 with 1 g hardener (dissolved within 5 min) on a shaker overnight (approx. 12 h). Samples were then transferred to Histoform S, orientated and the polymerization was initiated with 1 ml hardener 2 in 15 mL solution and embedded within five minutes. After the polymerization, blocking of the embedded specimen was carried out with Technovit 3040. Samples were cut to 2 µm slices with a rotary microtome (Jung RM 2065; Leica, Germany) transferred to microscope slides, and hematoxylin-eosin (HE) stained.

Gills were analysed at 400 x magnification with the PALM Robo Imaging Software and a Zeiss AxioObserver microscope attached to a CCD camera (Carl Zeiss MicroImaging GmbH, Germany). Within 5 primary filaments per sample a total of 100 secondary lamellae were considered for each fish and histopathological changes were recorded. Dorsal and ventral secondary lamellae were considered in same amounts. Histopathological changes of the secondary lamellae and interlamellar spaces of the primary filament in-between were recorded according to Monteiro, Rocha, Fontainhas-Fernandes & Sousa (2008).

Conversion of nitrate in stomach content of tilapia

To examine the potential conversion of nitrate *in vitro*, the stomach content (1.5 ml per fish) of adult tilapia (550-650 g, n=20) was collected after sacrifice. After centrifugation (16000 *g* for 2 min), nitrate stock solution (3.035 g $NaNO_3$ in 10 mL) was added to the supernatant (gastric juice) to reach a target concentration of 1000 mg L^{-1} NO_3^--N. Samples (gastric juice and solids)

were mixed gently with the tip of the pipette and incubated at room temperature for 5, 45, 90 and 150 min respectively. After incubation, samples were centrifuged (16000 g for 5 min) and supernatant was analyzed for NO_2^- and NO_3^- (mg L^{-1}-N) as described earlier.

Statistical analysis

Data are presented as means ± standard deviation (SD) of n samples. Statistical analysis was performed using Graphpad Prism (GraphPad Software Inc., La Jolla, USA). Data were tested for normality (Shapiro-Wilk) and equal variance (Kruskal-Wallis). Multiple comparisons were carried out by non-parametric Dunn's test ($p<0.05$). Results for gill histology were expressed in percent and, prior to statistics, transformed with an arcsine-square root transformation.

Results

Survival and growth performance

During the experiment, mortality was only observed in the highest treatment group (1000 mg L^{-1} NO_3^--N), where three fish died. No further analyses were carried out on these fish. There was a general decrease in the specific growth rate (SGR) observed with increasing NO_3^- concentration (Fig.1). Lowest SGR (1.1 % d^{-1} ± 0.1) was recorded at 1000 mg L^{-1} NO_3^--N, which was significantly lower compared to the control group ($P<0.01$, non-parametric Dunn`s). The SGR already decreased at 100 mg L^{-1} NO_3^--N group, though not significantly different from control fish. The feed conversion ratio (FCR) increased with increasing nitrate concentration (Fig.2). Again, only the FCR at 1000 mg L^{-1} NO_3^--N was significantly increased at 1.1 g g^{-1} ± 0.2 compared to the control ($P<0.01$, non-parametric Dunn`s).

Blood parameters

There was an increase in the NO_2^-- and NO_3^-- plasma concentrations with increasing nitrate concentration (Fig.3). The maximum increase in plasma concentration of NO_2^- (516 µM NO_2^- ± 284) and NO_3^- (22 µM ± 2.8) was found at an exposure of 1000 mg L^{-1} NO_3^--N ($P<0.01$, non-

parametric Dunn`s), but no statistical analysis was carried out due to low n in the highest treatment group.

Total hemoglobin concentration decreased with increasing NO_3^- concentration (Fig.4), lowest (3.5 g/dL ± 0.8) in the 1000 mgL^{-1} NO_3^--N group ($P<0.05$, non-parametric Dunn`s). Congruently, an increase of methemoglobin with increasing $NO_3^-$ concentration (Fig.4) was observed. The highest methemoglobin concentration (44 % ± 9) was recorded in the treatment group exposed to 1000 $mgL^{-1}$ $NO_3^-$-N ($P<0.05$, non-parametric Dunn`s)

Hepatosomatic index (HSI)

We observed an increase in HSI with increasing NO_3^- concentrations (Fig.5). The highest HSI (1.5 ± 0.5) was recorded at 1000 mgL^{-1} NO_3^--N, but no significant differences were detected ($p<$ 0.05, nonparametric Dunn`s).

Gill histology

Major abnormalities observed here were hyperplasia of epithelial cells, hyperplasia in cells between the lamellae, hypertrophy of pillar cells, clubbing, hypertrophy of epithelial cells, hypertrophy of mucus cells, fusion of secondary lamella and epithelial lifting (Tab.1). No significant differences were analyzed between treatments, but, as a trend, most abnormalities increased with increasing NO_3^- concentrations (Tab.1). Congruently, occurrence of undamaged secondary filaments decreased with increasing nitrate concentrations. Above 100 mgL^{-1} NO_3^--N less than 50% of the lamellae were undamaged compared to 62 % in the control. A strong increase of hyperplasia in epithelial cells as well as secondary lamella was recorded, particularly in the treatment group exposed to 1000 mgL^{-1} NO_3^--N. Hypertrophy of pillar cells was frequently observed (between 20 % at 1000 mg L^{-1} NO_3^--N and 56 % at 500 mg L^{-1} NO_3^--N), but revealed high individual variability. In contrast, hypertrophy of mucus and epithelial cell was very low

240 (<5 %), again irrespective of treatment. Clubbing was equally low (<10 %) irrespective of
241 treatment. Other abnormalities encompassed less then 5 % of the total damages.

242 **Conversion of nitrate in the stomach of tilapia**

243 We observed a significant conversion of nitrate in the stomach content of Nile tilapia ($p<0.01$,
244 nonparametric Dunn`s, n=5). Nitrite already increased after 45 min, but not significantly different
245 compared to 14 µM NO_2^- (± 2) after 5 min. After 90 min, a significant increase up to 74 µM NO_2^-
246 (±14) was observed ($p<0.01$, nonparametric Dunn`s, n=5). No further increase of nitrite was
247 observed after 150 min (Fig.6)

248 **Discussion**

249 The aim of this study was to investigate if chronic exposure to realistic nitrate concentrations
250 observed in RAS (10-1000 mg L^{-1} NO_3^--N) induces adverse effects on growth performance, feed
251 conversion or health status in juvenile Nile tilapia and to provide data on safe nitrate
252 concentrations in intensive RAS-based tilapia culture. Mortalities only occurred in the highest
253 treatment group, confirming that the range of concentrations chosen was adequate. Due to
254 coagulation, we did not consider these fish for blood analysis. Directly after sampling, brown
255 colored blood was recorded in fish of the highest treatment group confirming
256 methemoglobinemia in these fish.

257 Both, decreasing SGR and increasing FCR were observed with increasing ambient nitrate
258 concentrations. Still, significant differences to the control were only observed at 1000 mg L^{-1}
259 NO_3^--N. In several studies, reduced growth performance was indicative of inadequate water
260 quality in tilapia. For example, Shaw & Handy (2006) evaluated chronic copper toxicity in Nile
261 tilapia, reporting depression of SGR from 1.58 (control) to 1.2. More pronounced, El-Sherif &
262 El-Feky (2009) observed a drastic decrease of SGR from 1.16 (control) to 0.53 in tilapia
263 fingerlings during an experiment at pH 6. Although there are no data on chronic nitrate toxicity in

tilapia, reduced growth as well as increased feed conversion has been observed in other species. For example, van Bussel et al. (2012) reported a significant decrease of SGR from 1.6 to 0.45 with increasing nitrate concentration, as well as a significant increase of FCR from 1.07 to 3.80 in juvenile turbot (*Scophthalmus maximus*). In comparison to turbot (van Bussel et al., 2012), pikeperch (Schram, Roques, van Kuijk, Abbunk, van de Heul, de Vries, Bierman, van de Vis & Flik (2014, b) and catfish (Schram et al. 2014, a), results of our study suggest that tilapia is less sensitive, not surprisingly with regard to the habitat of the respective species. Here, a low feeding rate was chosen to assure an optimal water quality. Still, the decrease in SGR observed here is moderate and thus unexpectedly good with regard to the control. Congruently, feed conversion was significantly reduced at 1000 mg L^{-1} NO_3^--N with an FCR of 1.13 compared to 0.72 in the control. In a study on deleterious sub-lethal ammonia exposure (0.4 mg L^{-1} NH_3-N) to juvenile Nile tilapia, FCR increased from 1.5 (control) to 8 (El-Shafai, El-Gohary, Nasr, van der Steen & Gijzen 2004). Here, at an exposure of up to 500 mg L^{-1} NO_3^--N, neither SGR nor FCR were affected. Congruently, no effects on FCR and SGR were reported in pikeperch (*Sander lucioperca*) at nitrate concentrations up to 358 mg L^{-1} NO_3^--N (Schram et al., 2014 b).

As a conclusion, reduced growth performance and feed conversion could be a consequence of increased energy expenditure required to counteract adverse effects, for example conversion of methemoglobin as later on discussed. Alternatively, growth depression could also arise from nitrate-mediated modulation of the thyroid axis, since nitrate competes with the uptake of iodide in the thyroid (Ward, Kilfoy, Weyer, Anderson, Folsom & Cerhan 2010). Thereby, formation of thyroid hormones T3 and T4 would be reduced which in turn leads to reduced growth. Still, plasma nitrate observed was low and nitrite much higher, supporting the conclusion that the formation of MetHb and the subsequent energy expenditure is the primary cause of reduced growth and feed conversion observed here.

The concentration of nitrate in the plasma samples was well below concentrations in ambient water. Nitrite and nitrate concentrations increased with ambient nitrate concentrations of the rearing water, but, in contrast to Schram et al. (2014, a, b), nitrite exceeded the nitrate concentrations in the plasma about 27 fold. Therefore, it seems that there was an uptake of nitrate, whether active or passive, followed by a reduction of nitrate to nitrite within the body of tilapia.

Until today, the uptake of nitrate is still poorly understood, mainly due to the fact that most tissues represent a barrier preventing the passage of the large hydrated nitrate ion. In their study on nitrate toxicity to African catfish (*Clarias gariepinus*) Schram et al. (2014, a) concluded that the integument of the fish forms a significant barrier to waterborne nitrate. As a consequence, alternative routes for nitrate uptake are limited and uptake via the gills seems most plausible with regard to the direct contact with the ambient water as well as the importance in osmoregulation and ion uptake (Hwang 2009). However, a low permeability for nitrate through the gills was discussed in trout (Stormer et al. 1996) and has been reported in freshwater crayfish (Jensen 1996). In contrast, nitrite uptake has been described for the gills as well as the intestinal wall. For example, Grosell & Jensen (2000) documented nitrite passage over the intestinal/stomach wall of the European flounder and nitrite uptake in the stomach is very fast in rats (Bryan, Fernandez, Bauer, Garcia-Saura, Milsom, Rassaf, Maloney, Bharti, Rodriguez & Feelisch 2005). Additionally, nitrite and chloride compete for the active branchial chloride uptake mechanism in freshwater fish (Williams & Eddy, 1986), and since the chloride concentration in freshwater is low, the presence of nitrite can lead to massive nitrite accumulation in the plasma (Grosell & Jensen, 2000). Furthermore, low stability of nitrite suggests rather acetic conditions to prevent fast oxidation.

311 Consequently we hypothesized that uptake involves a reduction of nitrate to nitrite in the
312 stomach, prior to the actual passage of the intestinal wall. Such route would result in high plasma
313 nitrite, similar to those observed here. Therefore, we assessed the reduction of nitrate to nitrite in
314 stomach juice in an *in vitro* experiment. We demonstrate that nitrate is rapidly converted into
315 nitrite reaching a maximum of 74 µM NO_2^- after 90 min. Our findings strongly indicate that
316 conversion of nitrate to nitrite in the gastro-intestinal system of tilapia represents the most
317 probable uptake route. As a consequence, nitrate toxicity in tilapia is mainly a result of nitrate
318 reduction to nitrite and irreversible oxidation of hemoglobin to methemoglobin. Nevertheless,
319 nitrate is quite stable (~ 8 h, Webb, Patel, Loukogeorgakis, Okorie, About, Misra, Rashid, Miall,
320 Deanfield, Benjamin, MacAllister, Hobbs & Ahluwalia 2008) and anaerobic conversion of nitrate
321 to nitrite in the gut needs to be considered (Webb et al. 2008; Speijers & van den Brandt 2003;
322 Fanning 2000).
323 In this experiment, observations, which are typically attributed to nitrite toxicity, furthermore
324 confirm nitrite mediated intoxication. At 500 and 1000 mg L^{-1} NO_3^--N, formation of
325 methemoglobin was 22.5 % (± 14.1) and 43.9 % (± 9.3), respecitvely. At lower concentrations,
326 methemoglobin was low, ranging between 8.9 % and 16.5 %. Considering the actual nitrite
327 concentrations from 23.9 µM (0 mg L^{-1} NO_3^--N) to 65.3 µM (100 mg L^{-1} NO_3^--N) in the plasma,
328 counteracting mechanisms seem to restore homeostasis until an ambient concentration of at least
329 100 mg L^{-1} NO_3^--N. Here, methemoglobin reductase converts methemoglobin to hemoglobin and
330 restores functionality of red blood cells, but also represents a substantial energy expenditure
331 (Choury, Leroux & Kaplan, 1981). Therefore, a decrease in SGR is most likely a result of
332 increasing methemoglobin formation and its energy demanding recycling. The presence of
333 around 10% methemoglobin in the blood as observed between 0 mg L^{-1} NO_3^--N and 100 mg L^{-1}
334 NO_3^--N are within the range reported as basic level in other species (Kroupova, Machova &

335 Svobodova 2005; Wuertz et al. 2013). A visible indicator for severe methemoglobinemia is the
336 formation of brown colored blood, which in Nile tilapia is first observed at approximately 20 %
337 of methemoglobin with no other symptoms of toxcicity (Svobodova, Machova, Poleszczuk,
338 Huda, Hamackova & Kroupova 2005). Here, brown color was observed during sampling of the
339 highest treatment group at 33.9 % - 52.2 % methemoglobin. Levels above 50% methemoglobin
340 are considered threatening to fish (Bowser, Falls, Vanzandt, Collier, & Phillips 1983), which
341 clearly identifies NO_3^--N $\geq$ 1000 mg L^{-1} as intolerable for the rearing of juvenile Nile tilapia. We
342 further recorded a significantly elevated HSI (Fig.5) at 1000 mg L^{-1} NO_3^--N which indicates other
343 adverse effects on the liver. Since nitrite is an oxidizing agent this finding may indicate increased
344 oxidative stress, but further studies are needed. Still, detoxification mechanisms to cope with
345 oxidative stress as well as elevated nitrite include enhanced turnover by catalase and cytochrome
346 c oxidase (summarized by Kroupova et al. 2005), which often lead to increased liver metabolism
347 and, subsequently, liver size. These processes are energy demanding and will hence further
348 reduce growth performance and increase FCR.
349 As gills comprise the largest surface in direct contact with the surrounding water (Evans,
350 Piermarini & Choe 2005) and subsequently represent the organ most heavily exposed,
351 abnormalities such as fusion of the secondary lamellae have been regarded as defense mechanism
352 limiting the uptake of toxins (Reiser, Schroeder, Wuertz, Kloas & Hanel 2010). Although some
353 histopathological changes have been recorded in the gills, high individual variation was observed
354 here. With regard to the low brachial permeability of nitrate, such lower incidence of gill
355 abnormalities seems plausible. Nevertheless, a decreasing trend of undamaged secondary
356 filaments from the control group to the highest treatment group was recorded (Tab.1). We also
357 observed increased hyperplasia of the epithelial cells as well as cells of the secondary lamella in
358 the highest treatment group, which are typically regarded as mild responses to increase the

diffusion barrier towards toxins in the water, compared to strong ones such as fusion of the lamella.

To our knowledge this investigation is the first one demonstrating that high nitrate concentrations, realistic for commercial RAS, impact juvenile tilapia at high concentrations of 500 mgL^{-1} NO_3^--N and 1000 mgL^{-1} NO_3^--N. Thus, tilapia is relatively robust towards nitrate and subsequent nitrite toxification. Here, no significant impacts on growth performance, feed conversion and health status were observed between 10 mgL^{-1} NO_3^--N and 500 mgL^{-1} NO_3^--N. Once more, it has been shown, that tilapia is well suited for intensive RAS-based aquaculture, but nutrient management such as decoupled aquaponics can improve animal health and welfare and production effectiveness.

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Figure captions

Fig. 1: Specific growth rate (SGR, mean ± SD) in juvenile Nile tilapia *Oreochromis niloticus* after 30 d of exposure to 0, 10, 100, 500 and 1000 mg L^{-1} NO_3^--N. Significant differences to the control are indicated by an asterisk ($p<0.01$, non-parametric Dunn's). The number of samples is indicated on top of each column. SGR= (ln final weight−ln start weight)/days*100

Fig. 2: Feed conversion ratio (FCR, mean ± SD) in juvenile Nile tilapia *Oreochromis niloticus* after 30 d of exposure to 0, 10, 100, 500 and 1000 mg L^{-1} NO_3^--N. Significant differences to the control are indicated by an asterisk ($p<0.01$, non-parametric Dunn's). The number of samples is indicated on top of each column. FCR= dry weight feed/ (final wet weight – initial wet weight)

Fig. 3: Plasma NO_2^- and NO_3^- (mean ± SD) in juvenile Nile tilapia *Oreochromis niloticus* after 30 d of exposure to 0, 10, 100, 500 and 1000 mg L^{-1} NO_3^--N. Significant differences to the control are indicated by asterisk ($p<0.01$, non-parametric Dunn's). The number of samples is indicated on top of each column. No statistical analysis was conducted in the highest treatment group for plasma NO_3^- due to a low number of replicates.

Fig. 4: Hemoglobin and methemoglobin concentrations (mean ± SD) in the blood of juvenile Nile tilapia *Oreochromis niloticus* after 30 d of exposure to 0, 10, 100, 500 and 1000 mg L^{-1} NO_3^--N. Significant differences to the control are indicated by asterisk ($p<0.05$, non-parametric Dunn's). The number of samples is indicated on top of each column.

Fig. 5: Hepatosomatic index (HSI, mean ± SD) in juvenile Nile tilapia *Oreochromis niloticus* after 30 d of exposure to 0, 10, 100, 500 and 1000 mg L^{-1} NO_3^--N. No significant differences were detected ($p< 0.05$, nonparametric Dunn`s). The number of samples is indicated on top of each column. HSI = (liver weight / final weight of fish) *100

Fig. 6: Conversion of nitrate (nominal concentration: 1000 mg L^{-1} NO_3^--N) to nitrite in the gastric juice of Nile tilapia after incubation at room temperature. Presented are the means (± SD, n= 5).

Significant differences to the start of the incubation (after 5 min) are indicated by asterisks ($p<0.01$, non-parametric Dunn's)