Effects of chronic sub-lethal nitrite exposure at high water chloride concentration on Atlantic salmon (*Salmo salar,* Linnaeus 1758) parr

Running title: Effects of chloride on nitrite toxicity in parr

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25 **Abstract**

26 The present study examined the protective effects of water chloride (Cl^-) towards nitrite toxicity
27 in Atlantic salmon parr during 84-day long nitrite exposure. Effects on growth, histology, blood
28 indices and gene expression were studied at a fixed nominal Cl^- concentration of 200 mg L^{-1}
29 and at several water nitrite concentrations (0, 0.5, 2, 5 and 10 mg L^{-1} NO_2^--N). The specific
30 growth rate was significantly reduced during the first three weeks at a Cl:NO_2^--N ratio of 21:1,
31 suggesting the activation of coping mechanisms at the later stages of the experiment. No
32 significant effect of nitrite on gill histology and mortality was found. Nitrite accumulated in
33 plasma; however, a Cl:NO_2^--N ratio of 104:1 or higher prevented nitrite entry. The concentration
34 of NO_2^--N in plasma was significantly reduced at the end of the study, supporting the
35 hypothesis of coping mechanisms. Cystic fibrosis transmembrane conductance regulator (*cftr*)-
36 1 showed a significant up-regulation at highest nitrite concentration on day 22, and in three of
37 the highest exposure groups at the end of the experiment. Our findings suggest that a Cl:NO_2^-
38 -N ratio above 104:1 should be maintained through episodes of nitrite accumulation in water
39 during the production of Atlantic salmon parr.

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41 **Keywords:** Nitrite toxicity, Atlantic salmon, performance, chloride to nitrite ratio

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1. Introduction

Recirculating aquaculture systems (RAS) are increasingly being used in Atlantic salmon producing countries (Bergheim, Drengstig, Ulgenes & Fivelstad, 2009; Terjesen et al., 2013). In a well-functioning RAS for Atlantic salmon smolts, nitrite (NO_2^-) generated from ammonia is relatively quickly converted in the biofilter to the less toxic nitrate (Stormer, Jensen & Rankin, 1996). However, biofilters at the start-up phase or that are operated outside optimal water quality ranges (e.g. pH, temperature, dissolved oxygen, alkalinity, salinity and organic matters) can lead to NO_2^- accumulation in the system due to the slower cell division of nitrite oxidizing bacteria compared to ammonia oxidizers (Chen, Ling & Blancheton, 2006). Additionally, a poor nitrification performance due to changes in operational and environment conditions such as nitrogen loading (change feeding rate and in diet composition), water exchange rates, fluctuation in salinity and sanitary treatments (as antibiotics), as well as high densities and the deterioration in water quality can result in elevated NO_2^- concentration (Noble & Summerfelt, 1996; Svobodová et al., 2005; Emparanza, 2009; Mydland et al. 2010; Kinyage, Pedersen & Pedersen, 2019). Toxic NO_2^- concentrations for the fish can thus be reached, which is one of the most frequent non-infectious water quality issues in Chilean RAS facilities (Noble & Godoy, 2002; Emparanza, 2009). Furhermore, NO_2^- toxicity can severely compromise fish health and survival in RAS, mainly when chloride concentrations are low ($<$50-100 mg L^{-1}) (Svobodová et al., 2005).

Accumulation of NO_2^- in tissues can result in mass mortality of fish as several functions such as gas transport, ion regulation, and cardiovascular, endocrine and excretory processes are affected (Jensen, 2003; Svobodová et al., 2005). In addition, exposure to nitrite can also induce oxidative stress and antioxidant responses in juvenile Brazilian flounder (Maltez et al., 2018). Nitrite has affinity for the branchial chloride uptake mechanism, i.e. NO_2^- can replace Cl^- in the chloride/bicarbonate (Cl^-/HCO_3^-) gill transporters (Jensen, 2003). Therefore, whenever NO_2^- is present in ambient water, a part of the Cl^- uptake will be shifted to NO_2^- uptake, which also can lead to chloride depletion (Stormer et al., 1996). The role of sodium-

71 potassium-chloride-cotransporter (NKCC) and cystic fibrosis transmembrane conductance
72 regulator (CFTR) in branchial transport of Cl^- have been recently documented (Evans, 2008,
73 2011; Marshall & Singer, 2002). These transporting proteins, in addition to Cl^-/HCO_3^-
74 transporters, are important for maintaining chloride homeostasis in teleosts and could be
75 affected by increased concentrations of nitrite in the water.

76 The toxic effect of NO_2^- decreased with increasing salinity levels (Kir & Sunar, 2017).
77 Elevation of the ambient Cl^- concentration can protect against nitrite uptake and thus against
78 nitrite toxicity (Crawford & Allen, 1977; Eddy, Kunzlik & Bath, 1982; Jensen, 2003; Perrone &
79 Meade, 1977). Indeed, adding chloride salts to water is the single most important method for
80 protecting fish against nitrite toxicity (Jensen, 2003). Acute toxicity of nitrite also can trigger the
81 oxidation of Fe^{2+} to Fe^{3+} in the heme molecule of hemoglobin, which leads to methaemoglobin
82 formation and reduces the blood oxygen transport capacity since methaemoglobin does not
83 bind oxygen (Lewis & Morris, 1986; Jensen, 2003; Kroupova et al., 2008; Kroupová, Valentová,
84 Svobodavá, Sauer & Máchová, 2016). A visible symptom of high methemoglobin levels is a
85 brown color in the blood and gills (Lewis & Morris, 1986; Kroupová, Valentová, Svobodavá,
86 Sauer & Máchová, 2016), known as brown-blood disease (Timmons & Ebeling, 2007).

87 For RAS applications, Tucker and Robinson (1990) and Timmons and Ebeling (2007)
88 recommended to keep the chloride to nitrite-nitrogen ratio ($Cl:NO_2^-$-N; w/w) above 20:1 to avoid
89 nitrite toxicity in catfish, tilapia, and trout. Svobodová et al. (2005) found that unstable biofilter
90 function and insufficient chloride concentrations were the main causes of mortality in three
91 RAS for catfish, tilapia, and tench, at $Cl:NO_2^-$-N ratio between 12:1 and 83:1. These authors
92 concluded that the optimal $Cl:NO_2$-N ratio is likely higher than previously proposed (Timmons
93 & Ebeling, 2007; Tucker & Robinson, 1990), and that the evaluation of optimal $Cl:NO_2^-$-N ratios
94 should take into account differences between fish species, life stages and environmental
95 conditions. Salmonids are among the most sensitive fishes to NO_2^- (Lewis & Morris, 1986;
96 Eddy & Williams, 1986). In view of the protective effect of water chloride on NO_2^- toxicity,
97 monitoring and supplementation of this anion should be considered vital in RAS. In Norway

and Chile, 90% of the smolt production sites have a Cl^- concentration below 14 and 9 mg L^{-1}, respectively, in the raw inlet water to the facilities, i.e. in the potential RAS make-up water (Kristensen, Atland, Rosten, Urke & Rosseland et al., 2009). However, the Cl^- concentration in the raw inlet water can be quite variable among sites (Kristensen et al., 2009) and seasons (Terjesen et al., 2013). Hence, a higher Cl^- level in the RAS water can be achieved through the make-up water at some sites. Alternatively, at sites with low raw inlet water Cl^- concentration, RAS designed for a high reuse of water (e.g. >99% of total flow rate) can be utilized, and a higher Cl^- concentration than in the make-up water can be maintained by dosing Cl^- and monitoring conductivity.

Some studies have examined acute nitrite exposure in salmonids (Bowser, Wooster, Aluisio & Blue, 1989; Eddy et al., 1982; Williams & Eddy, 1989). However, our understanding on the effects of chronic exposure of salmonids to low NO_2^- concentrations (Doblander & Lackner, 1996; Kroupova et al., 2008; Wedemeyer & Yasutake, 1978), and on the effects of NO_2^- on branchial gene expression of Cl^- transporting proteins is limited. Furthermore, to our knowledge no long-term NO_2^- exposure experiments have been reported for Atlantic salmon, despite the increased production of smolts in RAS and higher risk of NO_2^- exposure in closed containment systems. Norwegian authorities' guidelines recommend that water nitrite (NO_2^-) in smolt production systems should not exceed 0.1 mg L^{-1} (FDIR, 2004). Still, the guidelines provide no reference to water chloride concentration, except for seawater where the limit is 0.5 mg L^{-1} (FDIR, 2004). Hence, a better understanding of the effects of chronic NO_2^- exposure on growth rate, survival, welfare indicators, and molecular physiology of Atlantic salmon, as well as of optimal $Cl:NO_2^-$-N ratios is crucial for farmers, technology suppliers, and legislators. The main objective of the present study is therefore to investigate how fish performance (i.e. growth and feed conversion) and health, in particular gill integrity (i.e. pathology and selected physiological and molecular parameters markers for chloride homeostasis), in Atlantic salmon parr are affected by chronic exposure to different NO_2- concentrations at a relatively high level of water chloride.

125

126 **2. Materials and methods**

127 2.1 Experimental fish

128 Atlantic salmon used in this experiment originated from the SalmoBreed strain (SalmoBreed,
129 Bergen, Norway). The fish were hatched in December 2009 at the Nofima research station in
130 Sunndalsøra (Norway). In May 2010, a total of 810 Atlantic salmon parr of 6.9 ± 0.12 g ind^{-1}
131 (SD) size were stocked in 15 flow-through water tanks with a capacity of 150 L each, with 54
132 individuals per tank. The fish were then subjected to a four-week acclimatization period prior
133 to nitrite exposure to adapt to the experimental tanks and were given a standard commercial
134 feed during this period (Ewos Micro 5, 1.7 mm, Ewos, Bergen, Norway). At the start of the
135 exposure (day 0) in June 2010, the fish were bulk weighed, counted, and two individuals were
136 sampled per tank (all 15 tanks sampled). The average individual weight at start of the
137 experiment was 16.5 ± 0.6 g ind^{-1}. The use of experimental animals, protocol and procedures
138 were reviewed and approved by the Norwegian Animal Research Authority through the permit
139 number 2651.

140

141 2.2 Experimental design

142 The exposure system was designed to maintain a constant Cl^- concentration at five
143 different concentrations of NO_2^--N, thereby producing a series of Cl:NO_2^--N ratios (Table 1). A
144 ground well freshwater source was used in the experiment. Water temperature during the
145 exposure was 12.5 ± 0.1°C. Details on the chemical composition of the water source can be
146 found in Terjesen et al. (2013). Due to the variability in chloride concentration in the ground
147 well water at Nofima Sunndalsøra increasing its level from spring to autumn (see Terjesen et
148 al., 2013), a flexible NaCl dosing system was needed to achieve constant Cl^- as the experiment
149 progressed. NaCl (analytical grade, cat. no. 167-923568, Fisher Scientific, Oslo, Norway)
150 supplementation was therefore adjusted according to the raw water Cl^- concentration and
151 conductivity. One of three NaCl stock solutions of varying concentrations was pumped (8-

152 channel Watson-Marlow pump, mod. 520 Du, Bredel, Wilmington, NC, USA) into a header-
153 tank to keep the Cl^- concentration at a nominal 200 mg L^{-1} within the five nitrite treatment mixing
154 tanks, each connected to 3 replicate fish tanks. In addition to the three different NaCl stock
155 solutions, Watson-Marlow pump marprene tubes were used to provide 14 possible NaCl
156 dosing levels. A calibration curve between water Cl^- concentration and conductivity was
157 established prior to the trial. Based on daily conductivity measurements, this relationship was
158 subsequently used each day to choose the appropriate NaCl stock solution and number of
159 tubes combination. The NaCl solution was pumped into a main header tank, which contained
160 an aquarium pump to ensure mixing, as well as diffusers for oxygenation; the header tank then
161 led to the five nitrite mixing tanks.

162 Four out of five treatment mixing tanks were supplemented with different flows of
163 $NaNO_2$ (analytical grade, cat. no. 162-424354, Fisher Scientific) stock solutions and were
164 pumped by four other channels in the Watson-Marlow pump. The following nominal NO_2^--N
165 treatment concentrations were established in the pre-tanks: 0.5, 2, 5 and 10 mg L^{-1} NO_2^--N. No
166 NO_2^- was pumped to the control group mixing tank. The small difference that may have resulted
167 in Na^+ water concentration between groups due to the Na^+ accompanying the NO_2^- anion (max.
168 difference of 3.3 mg L^{-1}) was not adjusted. However, the difference in Na^+ is small to that
169 already present in raw water during autumn in this water source (Terjesen et al., 2013).
170 Furthermore, the difference in NO_2^--N concentration between control and the nominal 10 mg
171 L^{-1} group was of several orders of magnitudes larger than the change in Na^+. Therefore, the
172 results were interpreted as being primarily due to nitrite and Cl: NO_2^--N ratios.

173 From the mixing tanks, pipes led the water to three experimental fish tanks per
174 treatment. Technical failures in the pump system interrupted the $NaNO_2$ supply for the nominal
175 0.5 mg L^{-1} NO_2^--N group at days 13 and 27, and for the nominal 5 mg L^{-1} NO_2^--N group at day
176 46; however, malfunctions were rectified within 24 hours. Water quality data obtained during
177 these occasions were not included in the statistical analyses.

178 The fish were fed 22-h continuously (with belt feeders) per day, using commercial diets
179 (Ewos Micro 15 and 30, 2.2 and 2.8 mm pellets size, respectively). Daily rations were

180 calculated according to feed intake, which was determined by using a feed waste collecting
181 system on each tank, and 20% overfeeding according to Helland, Grisdale-Helland & Nerland
182 (1996).

183 Water flow was set to a nominal 2.6 L min^{-1} per tank, and oxygen levels were kept
184 above 85% saturation by using pure oxygen diffusers in each mixing tank (Table 1). A light:
185 dark regime of 24L:0D was used throughout the trial.

186

187 2.3 Measurements and sampling

188 The dosing pump for delivery of exposure compounds ($NaNO_2$ and NaCl) was checked
189 daily, and the pump flows were measured three times a week. Fish tank flow was measured
190 each week. The pump tubes were replaced if the observed deviations exceeded 10% of the
191 desired flow. Water samples from tank outlets, as well as pre-tanks, were collected every
192 second or third day for analysis of water quality. Conductivity and temperature were measured
193 daily using a HQ40D meter connected to a Hach Intelli-CAL CDC401 Standard Conductivity
194 probe (Hach Lange, Düsseldorf, Germany). pH was measured twice a week using a Hach
195 PHC10101 electrode on the HQ40D meter, while oxygen was measured three to five times a
196 week using a Hach Intellical LDO outdoor sensor (LDO101-5).

197 Fish were sampled at the start of the exposure (day 0; n=2 per tank), at exposure week
198 3 (days 21–22; n=15 per tank), at week 7 (days 48–49; n=12 per tank), and at the final sampling
199 at week 12 (days 83–84; n=12 per tank). Survival was recorded on a daily basis.

200 Feeding was continuous until sampling activities started each day, with all fish being
201 bulk weighed and counted. A total of 10–11 fish per tank were collected at each sampling
202 event, except at the start of the trial where only 2 fish per tank were collected. Fish were
203 anesthetized using tricaine methane sulphonate (100 mg L^{-1}, MS-222, Argent Chemical
204 Laboratories, Redmond, WA, USA) and subsequently euthanized. Blood samples were taken
205 from the caudal vein from four fish per tank using vacutainers with 45 USP lithium heparin
206 (Sigma-Aldrich, St. Louis, M.O., USA). Plasma was separated (10 min at 3000 r.p.m., Allegra
207 6R centrifuge, Beckman, CA, USA) and stored at -80°C for later nitrite analysis. At the sampling

208 points at day 22 and 84, blood was also taken from another three fish per tank for immediate
209 analysis of pH, glucose and chloride concentrations using an ISTAT Portable Clinical Analyser
210 (Abbott Laboratories, Abbott Park, IL, USA). Individual weight and length were measured for
211 all sampled fish. Stomach and gut content of each fish was also weighed, for later correction
212 of tank individual weight. Heart index (CSI, cardio somatic index) and liver index (HSI, hepato
213 somatic index) were determined for one and four fish per tank at the start of the exposure and
214 other sampling points, respectively.

215 The second gill arch on the left side of the fish was sampled from one and four
216 individuals per tank at the start of the exposure and all other sampling points, respectively. Gill
217 samples were split in two; one piece was collected in cryo tubes and frozen in liquid nitrogen
218 for later gene expression analysis, whereas the other half gill arch was collected in liquid
219 scintillation vials with 10 mL phosphate-buffered formalin (4%, pH 7.2) formalin. Of the latter
220 samples, gills from three treatments (0, 0.5 and 10 mg L^{-1} nominal NO_2^--N, collected as
221 described above) were taken after 84 days of nitrite exposure and sent to the Norwegian
222 Veterinary Institute (Oslo, Norway) for histology analysis.

223

224 ## 2.4 Analytical methods

225 Water samples were analyzed for NO_2^--N using an automated analyzer (Flow Solution
226 IV, OI Analytical, College Station, TX, USA), according to U.S. E.P.A Method 353.2 (U.S.EPA,
227 1983). The chloride concentration in water samples collected at intervals varying between two
228 and 12 days was analyzed using a Hach Digital Titrator with the silver nitrate method 8207
229 (Hach).

230 Blood pH, Cl^-, and glucose (Glu) was analyzed using the I-STAT analyzer, with EC8+
231 cartridges (Abbott Laboratories). The obtained pH values were temperature-corrected to the
232 relevant experimental water temperature according to Roth and Rotabakk (2012).

233 Nitrite (NO_2^--N) in plasma was analyzed according to Miranda, Espey & Wink (2001),
234 based on the Greiss-reaction, but without the prior nitrate reduction step. Briefly, plasma
235 samples were first deproteinized using Nanosep 30K cartridges (Fischer Scientific) with a 30

236 kDa cut-off. Subsequently, 150 µL of the deproteinized plasma was added to a reaction mix of
237 150 µl SULF (sulfanilamide) and 150 µL NEDD (*N*-1-(naphthyl) ethylenediamine) in disposable
238 cuvettes, and left for 45 minutes before reading absorbance at 540 nm. Plasma samples were
239 run in duplicate and the NO_2^--N concentration was calculated from standard curves prepared
240 each analysis day from duplicate assays of five known concentrations of NO_2^--N ($r^2 > 0.998$ for
241 all curves). Urea concentration was also analyzed on the deproteinized plasma samples
242 according to Rahmatullah and Boyde (1980).

243 For evaluation of gill histology, tissues were embedded in paraffin, cut into 5 µm
244 sections after surface decalcification (decalcifying solution light, Sigma–Aldrich) and then
245 stained according to a standard haematoxylin-eosin (H&E) histological protocol. H&E stained
246 slides of gill tissues were subsequently evaluated for evidence of damage or tissue change.
247 The samples were given a gill histopathology score of either 0) no change; 1) minimal changes,
248 one focus of e.g. epithelial hyperplasia or sub-epithelial cell infiltration; 2) one additional
249 observation of change in the section; 3) three observations of e.g. hyperplasia per sample; and
250 4) severe changes.

251 For analysis of gene expression in gill tissues, total gill RNA was isolated from 12
252 individuals per treatment (three weeks exposure and at the end of the trial) using TRIzolTM
253 and Micro to Midi Kit and DNAse1 treatment (Invitrogen, MD, USA). Purity and quantity of the
254 isolated RNA was measured by spectrophotometry (Nanodrop® ND-1000 Spectrophotometer,
255 NanoDrop Technologies, Wilmington, DE, USA). For all samples, 0.5 mg total RNA was
256 reverse transcribed to cDNA using a 50:50 mix of random hexamer and oligo(dT) primers and
257 Taqman Gold RT-PCR kit (Applied Biosystems, CA, USA). All reactions were performed in
258 accordance with the manufacturer's protocol.

259 Primers for expression analysis were based on known Atlantic salmon sequences.
260 *nkcc1* (Genbank no. NM_001123683) primers (Forward: TCTGAATTCGAAAGCACCGC and
261 Reverse: TAAATGTCCGGCACAACTCG) were designed using the Vector NTI Advance 10
262 (Life technologies, MD, USA) and NetPrimer (PREMIER Biosoft, CA, USA) software. *cftr1*
263 (Genbank no. AF155237) primers (Forward: GAACCTTCTCCAATATGGTTGAAGAGGCAAG

264 and Reverse: GCACAGTTTTCCTTCCCCAACTCCTAAC), and primers against the internal
265 standard gene *18S rRNA* (Forward: GCCCTATCAACTTTCGATGGTAC and
266 Reverse:TTTGGATGTGGTAGCCGTTTCTC) also were used from Singer et al. (2002) and
267 Jørgensen, Hetland, Press, Grimholt & Gjøen et al. (2007), respectively. PCR products from
268 all primers were cloned using pGEM T-easy (Promega, WI, USA) and sequenced with Big Dye
269 Terminator chemistry and the ABI 3730 automated sequencer, both delivered by Applied
270 Biosystems.

271 Triplicate real-time qPCR reactions were performed using the Light cycler 480 and
272 SYBR Green chemistry (Roche, Switzerland) at the following thermal cycling conditions: 95°C
273 for 10min, followed by 45 cycles at 95°C for 15s, 60°C for 15s and 72°C for 15s. Specificity
274 was assessed by the melting curves and on EDTA stained agarose gel. Relative *nkcc1* or *cftr1*
275 mRNA was normalized to relative *18S rRNA* mRNA levels. The transcription ratios were tested
276 by using the Relative Expression Software Tool, REST, including exact PCR efficiency of each
277 amplicon according to Pfaffl, Horgan & Dempfle (2002).

278

279 2.5 Data treatment and statistics

280 Specific growth rates (SGR, % day^{-1}) between sampling points (t, days) were calculated
281 using the individual body weight at the first sampling point (BW1) and at the last sampling point
282 (BW2), according to the equation:

283 $SGR = (\ln BW2 - \ln BW1) \times 100 / t$

284 Individual body weight measurements, BW1 and BW2, were also used to calculate feed
285 conversion ratios for particular periods (FCR) following Helland et al. (1996):

286 $FCR = TFI / (BW2 – BW1)$

287 where TFI represents the total individual dry feed intake over the experimental period in
288 question.

289 Condition factor (CF) was calculated from the individual weight (W, g) and corresponding
290 length (L, cm):

291 $CF = W \times L^{-3} \times 100$

292 Hepato somatic index (HSI, %) was calculated according to the equation:

293 HSI = (Liver weight / W) x 100

294 Cardio somatic index (CSI, %) was calculated according to the equation:

295 CSI = (Heart weight / W) x 100

296 Fish tank was used as the statistical unit unless otherwise indicated. All data are presented as
297 mean ± SD, except for gene expression data that are presented as mean ± SEM.

298 To test for significant differences between exposure treatment means, one-way
299 ANOVAs were used, with exposure treatment as the main factor. If significant, Tukey's multiple
300 comparison tests were subsequently applied to evaluate which treatment differed from which.
301 For plasma nitrite, repeated measures ANOVAs were used, with the main factor being
302 exposure treatment and repeated measures being sampling point to test for effects of exposure
303 duration. Differences between treatments in gene expression of *nkcc* and *cftr1* were tested for
304 significance by the Pair Wise Fixed Reallocation Randomization Test (Pfaffl et al., 2002).

3. Results

3.1 Exposure conditions

The exposure system maintained nitrite in the fish tanks from approximately 90% to 106% of the intended nominal NO_2^--N concentration (Table 1). A reduction in the order of 14.7 ± 5.0% (mean ± SD of all treatments) was observed in the nitrite concentration between mixing tanks and the outlet of the fish tanks. The actual Cl:NO_2^--N ratios therefore varied between 101% and 108% of the nominal ratios (Table 1). There was no significant difference between the Cl:NO_2^--N ratios measured during the first three weeks, compared with ratios obtained in the remainder of the experiment ($p > 0.05$). During the study, the tank outlet pH increased significantly by up to 0.07 units ($p \leq 0.05$) with the NO_2^--N exposure concentration (Table 1).

3.2 Survival, growth and feed utilization indices

No treatment-related mortality was observed during the experiment. The mean individual weight across treatments at the end of the experiment was 80.9 ± 8.4 g ind^{-1} (n = 15 tanks). No significant differences in weight were detected between treatments ($p = 0.16$) (Fig. 1), although the three groups exposed to the highest nitrite concentrations showed the lowest body weight at the end of the trial. However, during the first periods of the experiment, significant differences in specific growth rate (SGR) were found between the start of the exposure and week 3. The highest SGR was observed in the control group (2.9 ± 0.2 %BW d^{-1}). The lowest SGR occurred in the group exposed to a nominal 10 mg L^{-1} NO_2^--N concentration at a Cl:NO_2^--N ratio of 21:1 (2.5 ± 0.1 %BW d^{-1}) (Table 2), representing a 16% growth rate reduction. Significant differences in SGR between treatments were also observed between day 22 and day 49 of the experiment. In contrast, final individual body weight, SGR ($p = 0.13$), accumulated feed intake ($p = 0.14$) or feed conversion ratio (FCR, feed:gain) ($p = 0.25$) had no significant differences throughout the entire experimental period. In addition, no significant differences were found in HSI, CSI or condition factor (data not shown).

332 3.3 Blood indices

333 Blood pH averaged 7.2±0.0, whereas chloride and glucose averaged 131 ± 1 mmol l^{-1}
334 and 6.0 ± 0.2 mg dL^{-1}, respectively, at the last sampling point at day 84. None of these blood
335 indices changed significantly with treatment at neither day 22 nor at day 84 (Table 3).

336 The plasma nitrite concentration was significantly affected by the experimental
337 treatments at experimental day 22 ($p < 0.01$) and at day 84 ($p < 0.001$) (Fig. 2). The 104:1
338 $Cl:NO_2^-$-N ratio in water did not result in a significant plasma nitrite concentration in relation to
339 the control group. At lower $Cl:NO_2^-$-N ratios (which correspond to the two highest water NO_2^--
340 N concentration treatments), a significantly increased plasma NO_2^--N was observed (Figure
341 2). Length of exposure significantly affected plasma NO_2^--N ($p < 0.05$) when sampling day was
342 included in the statistical analysis in a repeated measures design. Hence, although still
343 affected by the ambient nitrite concentration, the exposure resulted in a significantly lower
344 plasma NO_2-N at the end of the experiment compared with day 22.

345

346 3.4 Histology

347 Gill histology of the fish in the control group or of fish exposed to 0.5 or 10 mg L^{-1} NO_2^-
348 -N showed no significant effect on the histology score at day 84 of treatment ($p = 0.54$) (Fig.
349 3). Overall, none of the histology samples investigated had a gill histopathology score higher
350 than two.

351

352 3.5 Gene expression

353 Branchial *nkcc1* transcription was not affected by nitrite exposure after 22 days (Fig.
354 4A) but a significant *nkcc1* up-regulation was found in the 2 mg L^{-1} NO_2-N nominal exposure
355 group (104:1 ratio) at day 84. However, no dose-response relationship was apparent between
356 exposure concentration and *nkcc1* transcription. In contrast, branchial transcription of *cftr1* was
357 significantly affected by the experimental treatments (Fig. 4B). Only fish in the 10 mg L^{-1} NO_2^-
358 -N nominal exposure group (21:1, $Cl:NO_2^-$-N ratio) showed an up-regulation of *cftr1*
359 transcription ($p < 0.001$) at day 22. All groups except the lowest nitrite concentration were

found to be significantly up-regulated relative to the control group at day 84 ($p \leq 0.05$) (Fig. 4B).

4. Discussion

Despite the relatively high water nitrite concentrations used in the present study, i.e. up to 100 times the limit stated in water quality guidelines (FDIR, 2004), no treatment-related mortality occurred during the experiment. This observation is supported by the protective effects of water chloride on nitrite toxicity in teleosts (Bowser et al., 1989; Crawford & Allen, 1977; Jensen, 2003; Svobodová et al., 2005). However, little was known of the optimal $Cl:NO_2^-$-N ratio for Atlantic salmon during the parr stage under conditions promoting rapid fish growth in the control group. In this sense, the present study provides information as to which $Cl:NO_2^-$-N ratios are necessary to provide sufficient protection against adverse effects of chronic nitrite exposure during smolt production, under a fixed and high chloride concentration.

Low level chronic nitrite exposure is common in RAS for Atlantic salmon production. For instance, nitrite ranged from below 0.01 to 3.7 mg L^{-1} NO_2^--N in a facility producing 5 million smolts per year using RAS (monitored daily over 2.5 years, Frode Mathisen, Grieg Seafood, pers. com.). In addition, it has been reported that routine procedures such as removal of large water volume from the system can cause changes in water temperature and pH. These changes can consequently affect the nitrification process and lead to nitrite accumulation up to 6 mg L^{-1} in commercial RAS for salmonids in Chile (Emparanza, 2009).

High nitrite level in fish tank can be caused by many reasons such as improper feeding ration, antibiotics baths and poor nitrification performance, particularly in the its second stage, i.e. NO_2^- oxidation to NO_3^-. This condition has resulted in damage or mortality in different fish culture: catfish ($NO_2^- =$ 1.6 mg L^{-1}; $Cl^-:NO_2$-N ratios of 27.5), tench ($NO_2^- =$ 1.2-1.9 mg L^{-1}; $Cl^-:NO_2$-N= 11.8-18.8) and tilapia (0.8-2.0 mg L^{-1}; $Cl^-:NO_2$-N= 27-83), (Svobodová et al., 2005). Therefore, it was recommended that chloride concentrations should be raised to at least 100 mg L^{-1} as a preventive measure (Svobodová et al., 2005). Recently, it has been recommended

387 for cold water species, such as trout, chloride levels above 200 mg L^{-1} as designing criteria for
388 RAS operations (Timmons et al., 2018).

389 The trend in commercial RAS is to operate under high salinity levels for early stages of
390 salmon, to reduce stress levels and the amount of energy required for osmoregulation
391 (Timmons et al., 2018). In consequence, to avoid nitrite toxicity in RAS is recommended to
392 maintain a high Cl^- concentration (100-200 mg L^{-1}) (Svobodová et al., 2005; Timmons et al
393 2018). Hence, our experimental set up was defined to maintain a fixed nominal high Cl-
394 concentration of 200 mg L^{-1}. This is important for water inlets chloride concentration that can
395 fluctuate at seasonal scale (see Terjesen et al., 2013) as well as at spatial scale in Norway
396 and Chile (see Kristensen et al., 2009).

397 Outcomes from this study can serve as a guideline for Cl^- dosage in RAS, trying to
398 maintain a fixed high Cl^- level. For example, for a concentration of 1.9 mg L^{-1} NO_2^--N, more
399 than 200 mg L^{-1} of Cl^- must be the target at tank level to reach a NO_2^--N: Cl ratio above 104:1.
400 If the water inlet has a Cl^- concentration of 14 and 100 mg L^{-1}, then 186 and 100 mg L^{-1} of Cl^-
401 would be added, respectively. This concentration of Cl^- represents an applicable dosage of
402 307 and 165 mg L^{-1} of NaCl (analytical grade), the equivalent in grams of NaCl per m^{-3} of inlet
403 water, respectively. Even though, this Cl^- dosage can be considered an easy operational
404 routine to avoid nitrite toxic sub-lethal effects on salmon, the temporal variability of Cl- at the
405 inlet should be properly defined throughout long-term water quality monitoring programs.

406 The growth rate of the control fish group observed in the present study was comparable
407 with the growth rates observed in commercial salmon smolt production, with 76% to 130% of
408 that expected from tables based on industry growth rate data (Skretting, 2006, 2009). However,
409 specific growth rates of the exposed fish were significantly affected by nitrite during the first 22
410 days. The fish exposed to a nominal 10 mg L^{-1} NO_2^--N concentration, at a Cl:NO_2^--N ratio of
411 21:1, showed a significantly reduced growth rate compared with the control group. Kroupova
412 et al. (2008) reported that the specific growth rate of rainbow trout also was reduced when
413 exposed to Cl:NO_2^--N ratios below 33:1. The previously recommended water Cl:NO_2^--N ratio
414 of at least 20:1 for RAS (Timmons & Ebeling, 2007) is therefore not sufficient to protect Atlantic

415 salmon parr during the early phases of nitrite exposure. Instead, our findings suggest that a
416 $Cl:NO_2^-$-N ratio of at least 104:1 is necessary to avoid nitrite accumulation in Atlantic salmon
417 parr; this ratio should consequently be implemented as the new threshold.

418 The present results suggest that the cost of tissue maintenance or of growth was not
419 increased to any large extent by nitrite entry into the fish, since feed utilization (FCR) was not
420 significantly affected (Table 2). Nitrite might have adversely affected growth during the first
421 weeks of exposure by disturbing the oxygen carrying capacity or in growth-controlling parts of
422 the endocrine system; for instance, by transformation of nitrite to nitric oxide or by nitrite
423 replacing this hormone (Jensen, 2003). Indeed, it has been suggested that nitrite is not only
424 an inert molecule. Moreover, Bryan et al. (2005) demonstrated that NO_2^- can regulate gene
425 expression in mammalian tissues, and several observations indicate that nitrite can be
426 transformed to NO in rainbow trout erythrocytes (Jensen, 2006).

427 Regarding blood indices during nitrite exposure, in the study by Kroupova et al (2008),
428 no significant differences were found in methaemoglobin levels in blood, despite that significant
429 changes were found in several other parameters such as growth rate and plasma nitrite
430 concentrations (Kroupova, et al., 2008). Possibly, unlike nitrite in plasma, methaemoglobin
431 measurements are not sufficiently reflective of water nitrite concentrations during chronic sub-
432 lethal exposure, in contrast to the situation during acute exposure studies. Moreover, chloride
433 can be depleted during nitrite exposure (Jensen, 2003), and blood glucose has been shown to
434 increase in turbot (Jia et al., 2015); however, none of these responses were observed in the
435 present study. A response in these blood parameters cannot be ruled out however, for the very
436 first days of exposure, since the first sampling was done at 22 days. However, the results
437 suggest that unlike in rainbow trout (Kroupova, et al., 2008) and Atlantic cod (Siikavuopio &
438 Sæther, 2006), major ion-regulatory disturbances or hyperglycemia, typical of stressful
439 conditions in fish (Ackerman, et al., 2000; Pankhurst, 2011), are not chronically induced in
440 Atlantic salmon parr at the $Cl:NO_2^-$-N ratios used in the present study. Previous studies support
441 the lack of chloride depletion, as fish apparently maintain a fixed internal chloride concentration
442 even when nitrite is present in high levels (Lewis & Morris, 1986) and most blood parameters

443 of animals exposed subchronically to nitrite did not differ from the control treatment (Silva et
444 al., 2018).

445 Nitrite accumulated significantly in plasma of the salmon parr at the two highest
446 exposure concentrations, but concentrations were kept below that in the ambient water in fish
447 of all groups (Fig. 3). This agrees with the situation in rainbow trout chronically exposed to
448 nitrite, in which plasma levels were considerably lower than ambient water (Kroupova et al.,
449 2008). The same was observed when African catfish was exposed during 28 days to water
450 NO_2^--N concentrations up to 13 mg L^{-1} (Roques et al., 2015). The direction of the tissue:water
451 nitrite gradient is, however, dependent on the water chloride concentration, and there are both
452 inter- and intraspecific differences regarding the extent of nitrite tissue accumulation (Jensen,
453 2003). As an example, NO_2^--N levels in plasma of rainbow trout exposed to nitrite at a 3:1
454 $Cl:NO_2^-$-N ratio increased above the ambient water concentration during the first day of
455 exposure (Stormer et al., 1996). In the present study, the nitrite concentration in plasma was
456 significantly affected by length of exposure, such that at 84 days, plasma concentration had
457 decreased to 40 ± 9 % from values at day 22 (all exposure groups pooled). Thus, as also
458 witnessed by the lack of effect on growth rate in the last part of the trial, a potential adaptation
459 towards the ambient nitrite occurred in the Atlantic salmon parr. This may have occurred either
460 by reduction in the nitrite influx rate, increased excretion of nitrite, or by tissue detoxification
461 mechanisms and not specifically by the protective effects of chloride. Doblander and Lackner
462 (1996) showed that trout hepatocytes have the ability to oxidize nitrite to nitrate, and the
463 authors suggested that the sensitivity of fish towards nitrite is not only governed by branchial
464 uptake but also by detoxification systems in liver and other tissues. The ability of African catfish
465 to acclimatize to relatively high nitrite concentrations in water (up to 13 mg L^{-1} NO_2^--N) was
466 also attributed to internal detoxification of nitrite to less toxic nitrate (Roques et al., 2015). The
467 presence of uric acid increases nitrite oxidation to nitrate by a factor of two in the rainbow trout
468 hepatocytes (Doblander & Lackner, 1996). Therefore, further studies should combine
469 nucleotide rich feeds (e.g. Andersen et al., 2006) and nitrite exposure treatments in Atlantic

470 salmon since nucleic acid-rich diets increase plasma uric acid in salmon and rainbow trout to
471 20–44 µmol/L (Aas et al., 2006; Andersen et al., 2006).

472 In contrast to *nkcc1*, mRNA expression of the *cftr1* anion channel was responsive to
473 the experimental treatments. In this sense, *cftr1* may be considered a novel marker for nitrite
474 exposure in Atlantic salmon parr. In rainbow trout, the number of gill chloride cells correlate
475 with increasing water nitrite concentration during chronic exposure at a constant 10 mg L^{-1} Cl^-
476 concentration (Kroupova et al., 2008). The chloride cell number has also been found to
477 correlate with the nitrite concentration in plasma of trout (Krous, Blazer & Meade, 1982).
478 Moreover, gills were the preferred tissue for transcriptomics in response to acute nitrite toxicity
479 in bighead carp (Miao et al., 2018). Regarding the cellular location in gill tissues, the CFTR
480 protein is localized at the basolateral membrane of the chloride cells in freshwater adapted
481 killifish (Marshall & Singer, 2002); these authors concluded that the basolateral location is
482 consistent with CFTR involved in NaCl uptake in freshwater. In Atlantic salmon, *cftr1*
483 transcription increases rapidly at sea water transfer and is subsequently sustained (Singer et
484 al., 2002), although Stefansson et al. (2012) suggest a more complex regulation of CFTR
485 during sea water migration. Interestingly, *cftr1* mRNA expression is significantly up-regulated
486 by cortisol-implants in Atlantic salmon smolts (Singer et al., 2003). Taking into account that
487 nitrite increases chloride cell abundance, and that CFTR is involved in NaCl uptake, it is
488 hypothesized that the observed up-regulation of *cftr1* mRNA expression in salmon parr during
489 nitrite exposure might be associated with an increased number of chloride cells. Considering
490 that nitrite competes with Cl^- for transport across the gill, chloride cell proliferation during
491 exposure of the fish to nitrite may be a compensatory mechanism to maintain internal Cl^-
492 balance. Such a putative coping mechanism may explain why no significant chloride depletion
493 was detected in the present study, despite nitrite exposure resulted in elevated nitrite in
494 plasma. Future studies using an acute-type exposure model in Atlantic salmon parr may assist
495 in elucidating such a putative mechanism, as well as potential detoxification routes.

496 In conclusion, this study provides recommendations as to which chloride to nitrite ratios
497 can counteract certain adverse effects of nitrite during long-term exposure in Atlantic salmon

498 parr, based on growth rate, and physiological and molecular responses. It is suggested that
499 for smolt production, water quality must be maintained so that any nitrite present in the water
500 is not able to enter the fish via branchial channels. Measurements of nitrite in plasma are useful
501 in this regard and indicate that a Cl:NO_2^--N ratio in the water above 104:1 is necessary to avoid
502 nitrite accumulation in Atlantic salmon parr. Therefore, we suggest that during episodes of
503 nitrite accumulation in RAS for Atlantic salmon parr, or when such nitrite peaks can be
504 expected to occur, chloride should be added to the water to maintain a Cl:NO_2^--N ratio above
505 104:1 to protect against initial growth rate reduction and nitrite entry.
506
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516

517 **References**

518 Aas, T.S., Hatlen, B., Grisdale-Helland, B., Terjesen, B.F., Bakke-McKellep, A.M., & Helland,
519 S.J. (2006). Effects of diets containing a bacterial protein meal on growth and feed
520 utilisation in rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 261, 357–368.
521 https://doi.org/10.1016/j.aquaculture.2006.07.033
522 Andersen, Ø., Aas, T., Stugor, S., Takle, H., van Nes, S., Grisdale-Helland, B., Helland, S.J.,
523 & Terjesen, B.F. (2006). Purine-induced expression of urate oxidase and enzyme
524 activity in Atlantic salmon (*Salmo salar*). Cloning of urate oxidase liver cDNA from three

525 teleost species and the African lungfish *Protopterus annectens*. FEBS J, 273, 2839–
526 2850. https://doi.org/10.1111/j.1742-4658.2006.05288.x
527 Bergheim, A., Drengstig, A., Ulgenes, Y., & Fivelstad, S. (2009). Production of Atlantic salmon
528 smolts in Europe--Current characteristics and future trends. Aquacultural Engineering,
529 41, 46–52. https://doi.org/10.1016/j.aquaeng.2009.04.004
530 Bowser, P.R., Wooster, G.A., Aluisio, A.L., & Blue, J.T. (1989). Plasma chemistries of nitrite
531 stressed Atlantic salmon *Salmo salar*. Journal of the World Aquaculture Society, 20,
532 173–180. https://doi.org/10.1111/j.1749-7345.1989.tb00999.x
533 Bryan, N.S., Fernandez, B.O., Bauer, S.M., Garcia-Saura, M.F., Milsom, A.B., Rassaf, T.,
534 Maloney, R.E., Bharti, A., Rodriguez, J., & Feelisch, M. (2005). Nitrite is a signaling
535 molecule and regulator of gene expression in mammalian tissues. Nature Chemical
536 Biology, 1, 290–297. https://doi.org/10.1038/nchembio734
537 Chen, S., Ling, J., & Blancheton, J.P. (2006). Nitrification kinetics of biofilm as affected by
538 water quality factors. Aquacultural Engineering, 34, 179–197.
539 https://doi.org/10.1016/j.aquaeng.2005.09.004
540 Crawford, R.E., & Allen, G.H. (1977). Seawater Inhibition of Nitrite Toxicity to Chinook Salmon.
541 Transactions of the American Fisheries Society, 106, 105–109.
542 https://doi.org/10.1577/1548-8659(1977)106<105:SIONTT>2.0.CO;2
543 Doblander, C., & Lackner, R. (1996). Metabolism and detoxification of nitrite by trout
544 hepatocytes. Biochimica et Biophysica Acta (BBA) - General Subjects, 1289, 270–274.
545 Eddy, F., Kunzlik, P., & Bath, R. (1982). Uptake and loss of nitrite from the blood of rainbow
546 trout, *Salmo gairdneri* Richardson, and Atlantic salmon, *Salmo salar* L. in fresh water
547 and in dilute sea water. Journal of Fish Biology, 23, 105–116.
548 https://doi.org/10.1111/j.1095-8649.1983.tb02885.x
549 Eddy, F., & William, E. (1986). Nitrite and Freshwater Fish. Chemistry and Ecology, 3, 1-38.
550 https://doi.org/10.1080/02757548708070832

551 Emparanza, E.J.M. (2009). Problems affecting nitrification in commercial RAS with fixed-bed
552 biofilters for salmonids in Chile. Aquacultural Engineering, 41, 91–96.
553 https://doi.org/10.1016/j.aquaeng.2009.06.010
554 Evans, D.H. (2008). Teleost fish osmoregulation: what have we learned since August Krogh,
555 Homer Smith, and Ancel Keys. American Journal of Physiology-Regulatory Integrative
556 and Comparative Physiology, 295, R704-R713. https://doi.org/
557 10.1152/ajpregu.90337.2008
558 Evans, D.H. (2011). Freshwater Fish Gill Ion Transport: August Krogh to morpholinos and
559 microprobes. Acta Physiologica, 202, 349–359. https://doi.org/10.1111/j.1748-
560 1716.2010.02186.x
561 FDIR (2004). Directorate of Fisheries. Notes to regulations of 22. December 2004, no. 1785,
562 concerning management of aquaculture facilities (in Norwegian,
563 akvakulturdriftsforskriften).
564 Helland, S.J., Grisdale-Helland, B., & Nerland, S. (1996). A simple method for the
565 measurement of daily feed intake of groups of fish in tanks. Aquaculture, 139, 157–
566 163. https://doi.org/10.1016/0044-8486(95)01145-5
567 Jensen, F. (2003). Nitrite disrupts multiple physiological functions in aquatic animals.
568 Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology,
569 135, 9–24. https://doi.org/10.1016/S1095-6433(02)00323-9
570 Jensen, F.B. (2006). Physiological effects of nitrite: Balancing the knife's edge between toxic
571 disruption of functions and potential beneficial effects. In: Fish Physiology, Toxicology,
572 and Water Quality. Ninth International Symposium. United States Environmental
573 Protection Agency, Capri, Italy, pp. 119–132.
574 Jia R, Han C, Lei JL, Liu BL, Huang B, Huo HH, Yin ST (2015). Effects of nitrite exposure on
575 haematological parameters, oxidative stress and apoptosis in juvenile turbot
576 (*Scophthalmus maximus*). Aquat. Toxicol. 169, 1-9. https://DOI:
577 10.1016/j.aquatox.2015.09.016

578 Jørgensen, S., Hetland, D., Press, C., Grimholt, U., & Gjøen, T. (2007). Effect of early
579 infectious salmon anaemia virus (ISAV) infection on expression of MHC pathway genes
580 and type I and II interferon in Atlantic salmon (*Salmo salar* L.) tissues. Fish Shellfish
581 Immunology, 23, 576–588. https://doi.org/10.1016/j.fsi.2007.01.005
582 Kir, M., & Sunar, M. C. (2017). Acute Toxicity of Ammonia and Nitrite to Sea Bream, Sparus
583 aurata (Linnaeus, 1758), in Relation to Salinity. Journal of the World Aquaculture
584 Society, 49(3), 516–522. doi:10.1111/jwas.12448
585 Kinyage, J.P., Pedersen, P.B., & Pedersen, L.F. (2019). Effects of abrupt salinity increase on
586 nitrification processes in a freshwater moving bed biofilter. Aquacultural Engineering,
587 84, 91-98. https://doi.org/10.1016/j.aquaeng.2018.12.005
588 Kristensen, T., Åtland, Å., Rosten, T., Urke, H., & Rosseland, B.O. (2009). Important influent-
589 water quality parameters at freshwater production sites in two salmon producing
590 countries. Aquaculture Engineering, 41, 53–59.
591 https://doi.org/10.1016/j.aquaeng.2009.06.009
592 Kroupova, H., Machova, J., Piackova, V., Blahova, J., Dobsikova, R., Novotny, L., &
593 Svobodová, Z. (2008). Effects of subchronic nitrite exposure on rainbow trout
594 (*Oncorhynchus mykiss*). Ecotoxicology and environmental safety, 71, 813–820.
595 https://doi.org/10.1016/j.ecoenv.2008.01.015
596 Kroupová, H.K., Valentová, O., Svobodavá, Z., Sauer, P., Máchová, J. (2016). Toxic effects of
597 nitrite on freshwater organisms: a review. Reviews in Aquaculture, 0, 1–18. doi:
598 10.1111/raq.12184
599 Krous, S.R., Blazer, V.S., & Meade, T.L. (1982). Effect of Acclimation Time on Nitrite
600 Movement Across the Gill Epithelia of Rainbow Trout: The Role of “Chloride Cells”. The
601 Progressive Fish-Culturist, 44, 126–130. https://doi.org/10.1577/1548-
602 8659(1982)44[126:EOATON]2.0.CO;2
603 Lewis, W.M., & Morris, D.P. (1986). Toxicity of Nitrite to Fish: A Review. Transactions of the
604 American Fisheries Society, 115, 183–195. https://doi.org/10.1577/1548-
605 8659(1986)115<183:TONTF>2.0.CO;2

606 Maltez, L.C., Barbas, L.A.L., Nitz, L.F., Pellegrin, L. M., Okamoto, H., Sampaio, L.A.,
607 Monserrat, J.M., & Garcia, L. (2018). Oxidative stress and antioxidant responses in
608 juvenile Brazilian flounder Paralichthys orbignyanus exposed to sublethal levels of
609 nitrite. Fish Physiol. Biochem, 44, 1349–1362.
610 Marshall, W., & Singer, T.D. (2002). Cystic fibriosis transmembrane conductance regulator in
611 teleost fish. Biochimica et Biophysica Acta, 1566, 16–27.
612 Miao, L.H., Lin, Y., Pan, W.J., Huang, X., Ge, X.P., Zhou, Q.L., Liu, B., Ren, M.C., Zhang,
613 W.X., Liang, H.L., Yu, H., Ji, K. (2018). Comparative transcriptome analysis reveals the
614 gene expression profiling in bighead carp (Arisichthys nobilis) in response to acute
615 nitrite toxicity. Fish & Shellfish immunology, 79: 244-255
616 Miranda, K., Espey, M., & Wink, D. (2001). A rapid, simple spectrophotometric method for
617 simulataneous detection of nitrate and nitritie. Nitric Oxide, 5, 62–71.
618 https://doi.org/10.1006/niox.2000.0319
619 Mydland, L., Rud, I., Rudi, K., Ulgenes, Y., Ibieta, P., Gutierrez, X., Reiten, B., Summerfelt, S.,
620 Terjesen, B., (2010). Water quality and microbial community shifts during start-up,
621 disturbances and steady-state in a new moving bed bioreactor. In: Aquaculture Europe
622 2010, Porto, Portugal.
623 Noble, A.C., & Summerfelt, S.T., 1996. Diseases encountered in rainbow trout cultured in
624 recirculating systems. Annual Review of Fish Diseases 6, 65–92.
625 Noble, A., & Godoy, M. (2002). Enfermedades no infecciosas en sistemas de recirculación,
626 Parte I [Non-infectious diseases in recirculation systems. Part I]. AquaNoticias. 14: 65-
627 67.
628 Perrone, S.J., & Meade, T.L. (1977). Protective effect of chloride on nitrite toxicity to coho
629 salmon (*Oncorhynchus kisutch*). Journal of the Fisheries Research Board of Canada,
630 34, 486–492. https://doi.org/10.1139/f77-079
631 Pfaffl, M.W., Horgan, G.W., & Dempfle, L. (2002). Relative expression software tool (REST©)
632 for group-wise comparison and statistical analysis of relative expression results in real-
633 time PCR. Nucleic Acids Research, 30, e36-.

634 Rahmatullah, M., & Boyde, T.R.C. (1980). Improvements in the determination of urea using
635 diacetyl monoxime; methods with and without deproteinisation. Clinica Chimica Acta,
636 107, 3–9.
637 Roques, J.A.C., Schram, E., Spanings, T., Van Schaik, T., Abbink, W., Boerrigter, J., De Vries,
638 P., Van de Vis, H., & Flik, G. (2015). The impact of elevated water nitrite concentration
639 on physiology, growth and feed intake of African catfish *Clarias gariepinus* (Burchell
640 1822). Aquaculture Research, 46, 1384–1395. https://doi.org/10.1111/are.12292
641 Roth, B., Rotabakk, B.T., 2012. Stress associated with commercial longlining and recreational
642 fishing of saithe (Pollachius virens) and the subsequent effect on blood gases and
643 chemistry. Fisheries Research. 115–116, 110-114. https:// DOI:
644 10.1016/j.aquatox.2015.09.016
645 Silva, MJS., Costa, FFB., Leme, FP., Takata, R., Costa, DC., Mattioli, CC., Luz, RK., Miranda-
646 Filho, KC. (2018). Biological responses of Neotropical freshwater fish *Lophiosilurus*
647 *alexandri* exposed to ammonia and nitrite. Sci Total Environ. 616–617:1566–1575. doi:
648 10.1016/j.scitotenv.2017.10.157
649 Singer, T., Clements, K., Semple, J., Schulte, P., Bystriansky, J., Finstad, B., Fleming, I., &
650 McKinley, R. (2002). Seawater tolerance and gene expression in two strains of Atlantic
651 salmon smolts. Canadian Journal of Fisheries and Aquatic Sciences, 59, 125–135.
652 https://doi.org/10.1139/f01-205
653 Singer, T.D., Finstad, B., McCormick, S.D., Wiseman, S.B., Schulte, P.M., & McKinley, R.S.
654 (2003). Interactive effects of cortisol treatment and ambient seawater challenge on gill
655 Na^+,K^+-ATPase and CFTR expression in two strains of Atlantic salmon smolts.
656 Aquaculture, 222, 15–28. https://doi.org/10.1016/S0044-8486(03)00099-1
657 Skretting (2006). The Norwegian feed catalogue 2006 (In Norwegian). Skretting, Stvanger,
658 Norway.
659 Skretting (2009). The Norwegian feed catalogue 2009 (In Norwegian). Skretting, Stvanger,
660 Norway.

661 Stefansson, S.O., Haugland, M., Björnsson, B.T., McCormick, S.D., Holm, M., Ebbesson,
662 L.O.E., Holst, J.C., & Nilsen, T.O. (2012). Growth, osmoregulation and endocrine
663 changes in wild Atlantic salmon smolts and post-smolts during marine migration.
664 Aquaculture, 362–363, 127–136. https://doi.org/10.1016/j.aquaculture.2011.07.002
665 Stormer, J., Jensen, F.B., & Rankin, J.C. (1996). Uptake of nitrite, nitrate, and bromide in
666 rainbow trout, (Oncorhynchus mykiss): effects on ionic balance. Canadian Journal of
667 Fisheries and Aquatic Sciences, 53, 1943–1950. https://doi.org/10.1139/cjfas-53-9-
668 1943
669 Svobodová, Z., Máchová, J., Poleszczuk, G., Hòda, J., Hamáâková, J., & Kroupová, H. (2005).
670 Nitrite poisoning of fish in aquaculture facilities with water-recirculating systems. Acta
671 Veterinaria Brno, 74, 129–137. https://doi.org/ 10.2754/avb200574010129
672 Terjesen, B.F., Summerfelt, S.T., Nerland, S., Ulgenes, Y., Fjæra, S.O., Megård Reiten, B.K.,
673 Selset, R., Kolarevic, J., Brunsvik, P., Bæverfjord, G., Takle, H., Kittelsen, A., & Åsgård,
674 T. (2013). Design, dimensioning, and performance of a research facility for studies on
675 the requirements of fish in RAS environments. Aquacultural Engineering, 54, 49–63.
676 https://doi.org/10.1016/j.aquaeng.2012.11.002
677 Timmons, M., & Ebeling, J. (2007). Recirculating Aquaculture. Cayuga Aqua Ventures, Ithaca,
678 NY.
679 Timmons, M.B., Guerdat, T., & Vinci, B.J. (2018). Recirculating Aquaculture (4th edition).Ithaca
680 Publishing Company. Ithaca, NY, 779 p.
681 Tucker, C., & Robinson, E. (1990). Channel catfish farming handbook. Chapman & Hall, New
682 York, NY, USA.
683 Wedemeyer, G.A., & Yasutake, W.T. (1978). Prevention and Treatment of Nitrite Toxicity in
684 Juvenile Steelhead Trout (Salmo gairdneri). Journal of the Fisheries Research Board
685 of Canada, 35, 822–827. https://doi.org/10.1139/f78-132
686 Williams, E.M., & Eddy, F.B. (1989). Effect of nitrite on the embryonic development of Atlantic
687 salmon (*Salmo salar*). Canadian Journal of Fisheries and Aquatic Sciences, 46, 1726–
688 1729. https://doi.org/10.1139/f89-218

689

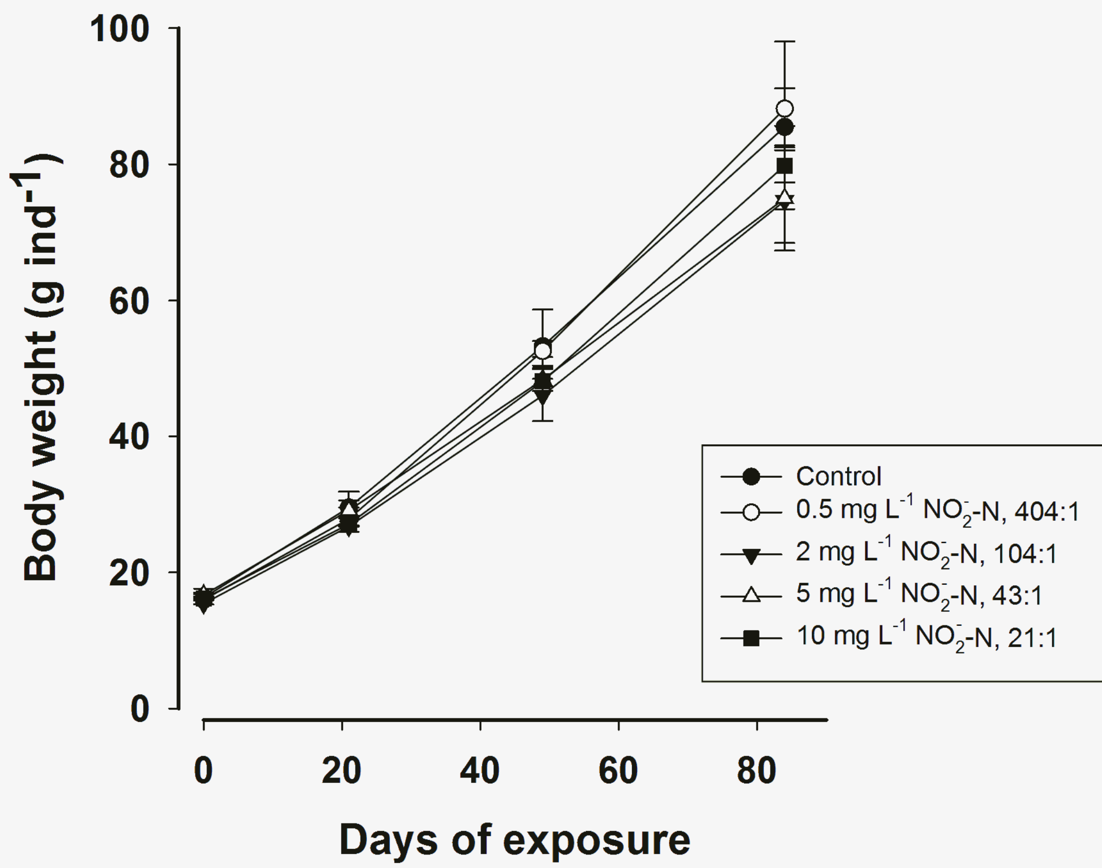
Figure legends

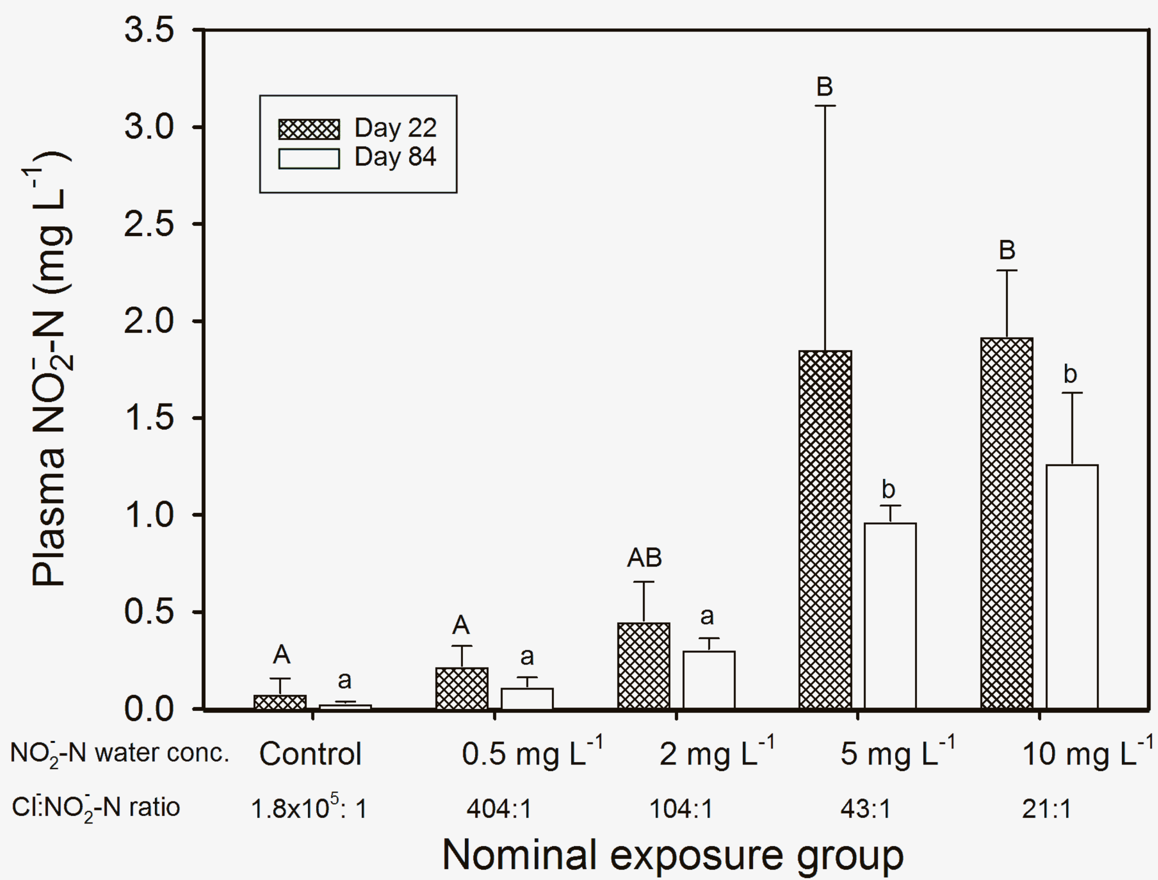
Fig. 1. Individual body weight of Atlantic salmon parr during the nitrite exposure study. Each data point indicates the tank mean (± SD) of each treatment (n = 3 tanks).

Fig. 2. Plasma NO_2^--N concentration in Atlantic salmon parr sampled after 22 and 84 days of NO_2^--N exposure. Each bar represents the mean (+ SD) of three samples; each sample consists of plasma pooled from four individual fish per tank. Significant differences ($p \leq 0.05$) are indicated by letters above the bars.

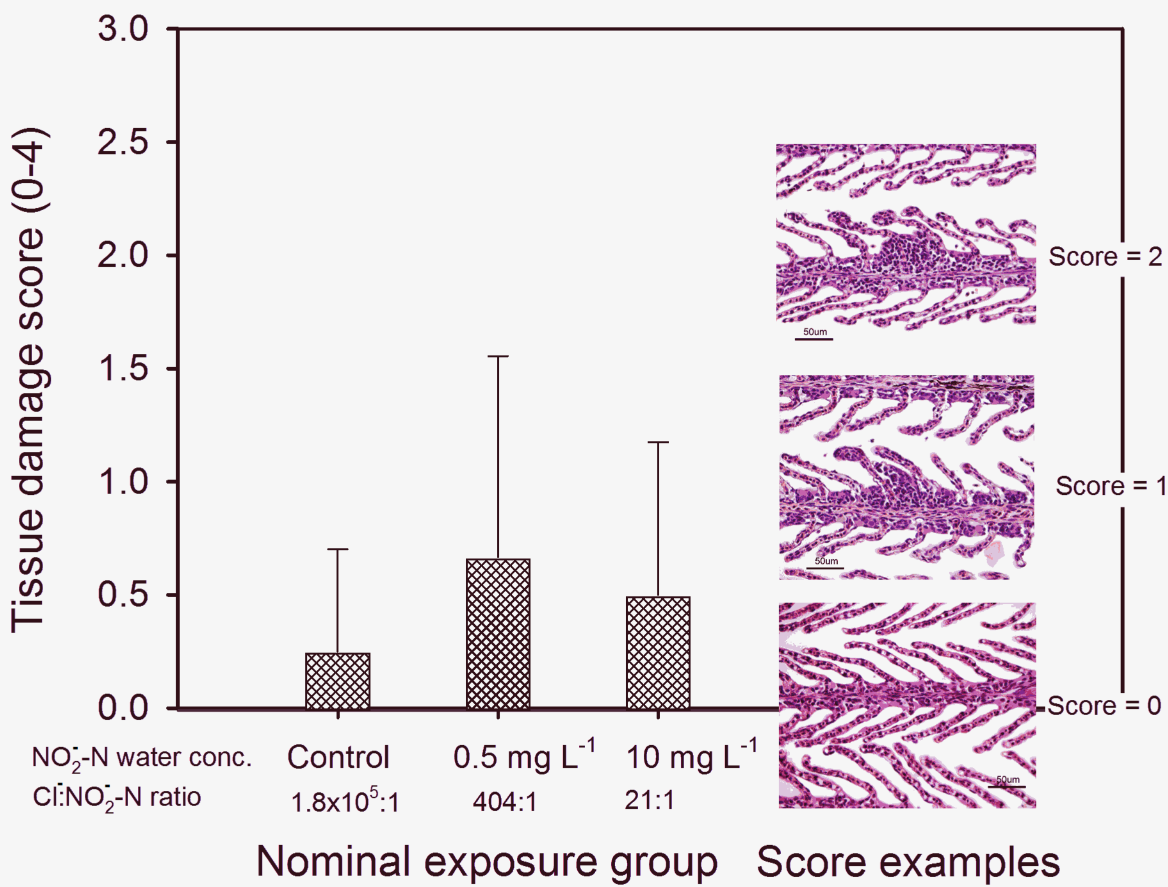
Fig. 3. Histological evaluation of gill tissues of Atlantic salmon parr when exposed to water nitrite for 84 days. Each bar indicates the mean score (+ SD) of three tanks; each tank value averaged from histology of four fish (12 fish per treatment). To the right, examples of scores 0, 1, and 2; scores greater than 2 were not observed.

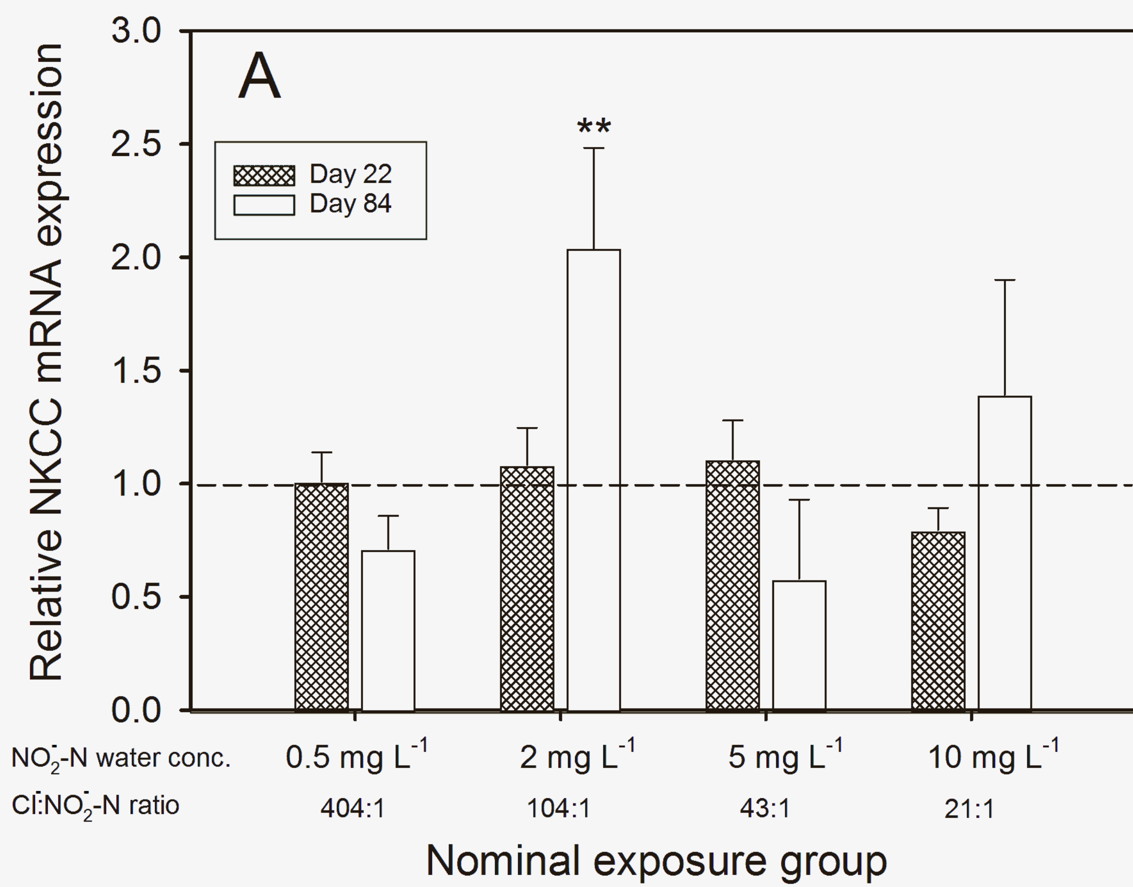
Fig. 4. NKCC1 (A) and CFTR1 (B) gill gene expression in Atlantic salmon parr sampled at 22 days (cross-hatched bars) and 84 days (open bars) since the start of the nitrite exposure. The data is normalized to the expression level in the control group (expression = 1, dashed horizontal line). Each bar indicates the mean (+ S.E.) values of 12 individuals per treatment. Differences are significant at $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; and $^{***}p \leq 0.001$.


Body weight (g ind^{-1})
100
80
60
40
20
0
0
20
40
60
80
Days of exposure
Control
0.5 mg L^{-1} NO_2^--N, 404:1
2 mg L^{-1} NO_2^--N, 104:1
5 mg L^{-1} NO_2^--N, 43:1
10 mg L^{-1} NO_2^--N, 21:1

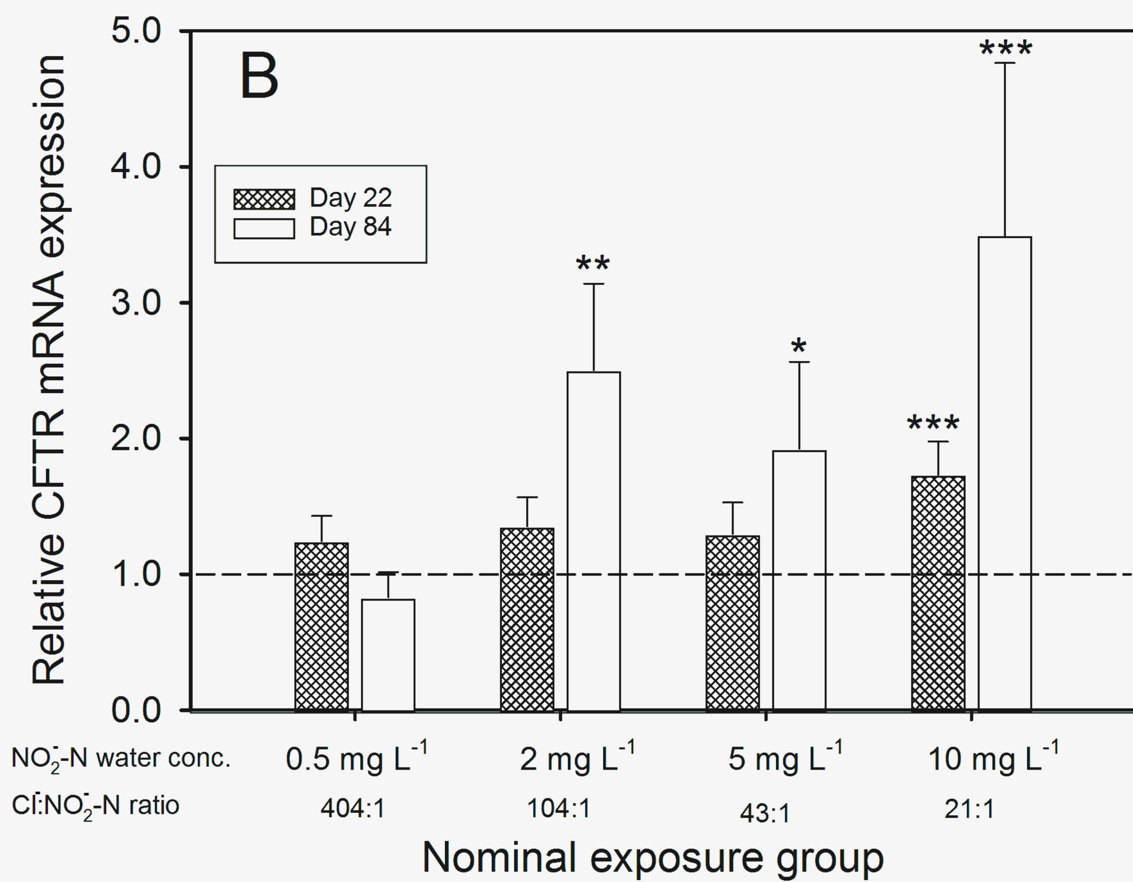


Plasma NO_2^--N (mg L^{-1})
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Day 22
Day 84
A
a
A
a
AB
a
B
b
B
b
NO_2^--N water conc.
Control
0.5 mg L^{-1}
2 mg L^{-1}
5 mg L^{-1}
10 mg L^{-1}
Cl^-:NO_2^--N ratio
$1.8x10^5$: 1
404:1
104:1
43:1
21:1
Nominal exposure group


Tissue damage score (0-4)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Score = 2
Score = 1
Score = 0
50um
NO_2^--N water conc.
Cl:NO_2^--N ratio
Control
$1.8x10^5$:1
0.5 mg L^{-1}
404:1
10 mg L^{-1}
21:1
Nominal exposure group
Score examples



A
Relative NKCC mRNA expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Day 22
Day 84
**
NO2-N water conc.
Cl:NO2-N ratio
0.5 mg L-1
2 mg L-1
5 mg L-1
10 mg L-1
404:1
104:1
43:1
21:1
Nominal exposure group



B
Day 22
Day 84
Relative CFTR mRNA expression
5.0
4.0
3.0
2.0
1.0
0.0
***
**
*
***
NO2-N water conc.
Cl:NO2-N ratio
0.5 mg L-1
2 mg L-1
5 mg L-1
10 mg L-1
404:1
104:1
43:1
21:1
Nominal exposure group

Table 1. Nominal and actual exposure concentrations of nitrite (NO_2-N) and chloride (Cl^-), and other conditions, during the 84 day long nitrite exposure study on Atlantic salmon parr.

Nominal exposure (NO_2-N, mg L^{-1})	[NO_2-N] (mg L^{-1})*	[Cl^-] (mg L^{-1})	Cl:NO_2-N (w:w)	Conductivity (µS cm^{-1})	Tank water flow (L min^{-1})	Water temperature (°C)	O_2 saturation (%)	pH
0	0.0 ± 0.0[a]	189.0 ± 22.9	$1.8 \times 10^5 \pm 0.9 \times 10^5$[§]	701 ± 63[a]	2.6 ± 0.1	12.4 ± 0.1	92.2 ± 1.3[b]	7.0 ± 0.1
0.5	0.5 ± 0.2[b]	187.2 ± 21.7	404 ± 179[d]	708 ± 62[a]	2.6 ± 0.1	12.5 ± 0.1	95.4 ± 0.9[ab]	7.0 ± 0.1
2	1.9 ± 0.4[c]	190.0 ± 23.0	104 ± 27[c]	726 ± 70[b]	2.5 ± 0.2	12.5 ± 0.1	99.9 ± 0.8[a]	7.1 ± 0.1
5	4.6 ± 0.6[d]	189.3 ± 22.3	43 ± 9[b]	749 ± 65[c]	2.6 ± 0.1	12.5 ± 0.1	92.9 ± 0.6[b]	7.1 ± 0.1
10	9.0 ± 1.0[e]	189.1 ± 23.8	21 ± 5[a]	785 ± 69[d]	2.6 ± 0.1	12.5 ± 0.1	93.1 ± 3.8[b]	7.1 ± 0.1

*All values in the table are the mean ± SD of measurements in the tank outlets (n=3 tanks per treatment), throughout the experiment. See text for details on sampling frequency during the trial. Tank means with differing letters are significantly different ($p \leq 0.05$, n=15).

[§]The control group Cl:NO_2-N ratios were not included in this statistical test, since the background levels of nitrite (below or around detection limits of the analysis) produced high variances in the ratios.

Table 2. Specific growth rate (SGR) and feed conversion ratio (FCR) of the Atlantic salmon parr during the 84 day long nitrite exposure study.

Nominal exposure (NO_2-N, mg L^{-1})	Cl:NO_2-N (w:w)	SGR day 1-22 (% day^{-1}) *	SGR day 22-43 (% day^{-1}) *	SGR day 43-84 (% day^{-1})	FCR day 22-43 (feed:gain)	FCR day 43-84 (feed:gain)
0	$1.8 \times 10^5 \pm 0.9 \times 10^5$	2.91 ± 0.24^{a}	2.08 ± 0.13^{ab}	1.33 ± 0.17	0.73 ± 0.02	0.77 ± 0.04
0.5	404 ± 179	2.59 ± 0.09^{ab}	2.25 ± 0.09^{a}	1.43 ± 0.13	0.73 ± 0.06	0.84 ± 0.05
2	104 ± 27	2.62 ± 0.02^{ab}	1.90 ± 0.20^{ab}	1.35 ± 0.19	0.77 ± 0.05	0.78 ± 0.03
5	43 ± 9	2.73 ± 0.10^{ab}	1.79 ± 0.17^{b}	1.24 ± 0.29	0.82 ± 0.06	0.85 ± 0.09
10	21 ± 5	2.47 ± 0.14^{b}	2.02 ± 0.04^{ab}	1.41 ± 0.07	0.77 ± 0.04	0.81 ± 0.02

*All values in the table are the mean ± SD of measurements (n=3 tanks per treatment), throughout the experiment. Tank means not sharing similar letters are considered significantly different ($p \leq 0.05$, n=15).

Table 3. Blood chloride (Cl^-), pH and glucose (Glu) of the Atlantic salmon parr at day 22 and 84 of exposure to different NO_2-N concentration.

Experimental day	Nominal NO_2-N exposure (mg L^{-1})	Cl:NO_2-N ratio	Cl^- (mmol l^{-1})*	pH*	Glu (mg d^{l-1}) *
22	0	1.8x10^5	121.2 ± 3.5	7.2 ± 0.0	5.4 ± 0.4
22	0.5	404	112.6 ± 4.7	7.3 ± 0.1	4.8 ± 0.9
22	2	104	122.0 ± 2.3	7.1 ± 0.2	5.5 ± 0.2
22	5	43	114.4 ± 1.3	7.2 ± 0.1	5.3 ± 0.5
22	10	21	113.2 ± 5.7	7.2 ± 0.0	4.6 ± 0.4
84	0	1.8x10^5	130.1 ± 0.6	7.2 ± 0.0	5.9 ± 0.7
84	0.5	404	131.0 ± 1.1	7.2 ± 0.0	6.1 ± 0.5
84	2	104	131.6 ± 2.8	7.3 ± 0.0	5.9 ± 0.4
84	5	43	129.9 ± 2.6	7.2 ± 0.1	6.3 ± 0.9
84	10	21	132.7 ± 2.4	7.2 ± 0.0	5.8 ± 0.3

*All values in the table are the mean ± SD of blood parameter measurements (n=9 fish per treatment), throughout the experiment. See text for details on sampling frequency during the trial.