Survey of histopathological effects and evaluation of performance in juvenile zebrafish (*Danio rerio*) under chronic exposure to nitrate

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

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"The right man in the wrong place can make all the difference in the world.

So wake up. Wake up and smell the ashes"

(G-Man *in* Half-Life 2)

Abstract

Danio rerio (Hamilton, 1822) is one of the biological research models currently in vogue. Despite its enormous importance in various fields of science and biomedicine, its rearing is not properly standardized and is based on observations and on conditions used in other aquaculture species. To ensure reproducible and solid results, standardization of the rearing conditions for this species has to be ensured and studies must be held to define the ideal growing conditions. One of the rearing conditions not set to the zebrafish is the concentration of nitrate. Nitrate is considered by many as nontoxic as the fish have increased resistance to this ion than to the ammonia and nitrite ions, so it is often neglected. However, in water recirculation systems (RAS) nitrate can reach high values. This study aims to determine the effects of chronic exposure to nitrate in zebrafish tissues and performance.

With this proposed, juvenile zebrafish were exposed to concentrations of 0, 100, 200 and 400 mg/L NO_3^--N for 28 days. As $NaNO_3$ was used as the source of nitrate, zebrafish juveniles were also exposed to concentration of 1.7 g/L NaCl in order to equalise the concentrations of salts of the higher concentration of $NaNO_3$. Also, in order to evaluate whether the accumulated ammonia could influence fish performance in the static systems, juveniles were also placed in a water recirculation system. At the end of exposure, fish were euthanized, weighed, fixed and processed for histological analysis using conventional techniques. Histopathological effects were measured using the semi-quantitative system proposed by Bernet et al. (1999) and modified by Saraiva et al. (2015).

The results obtained clearly indicate that chronic exposure of juveniles of *D. rerio* to nitrate concentrations higher than 200 mg/L NO_3^--N caused deleterious effects in growth performance and health status. It was also stated that the histological changes observed in gills, skin, kidney and intestine were mainly caused by nitrate concentration but the ones observed in liver were caused mainly by ionic concentration. The main histopathological phenomena observed in gills were oedema, hyperaemia, haemorrhages, hyperplasia and necrosis; in skin were epidermis and in some cases also dermis desquamation due to necrosis; in kidney were vacuolar degeneration of renal tubules epithelial cells, hyperaemia and haemorrhage, deposits in renal tubules lumen, some renal tubule atrophy and necrosis of the haematopoietic interstitial tissue; in liver were hepatocyte vacuolation, hypertrophy, necrosis and depletion of lipid reserves; in intestine were vacuolation and hypertrophy on the of the enterocytes of the posterior intestine, goblet cells hyperplasia in the anterior intestine and villi atrophy.

It is hoped that these findings will contribute to the standardization of the zebrafish rearing conditions and might be taken into account in future studies.

Keywords

Zebrafish, nitrate, chronic toxicity, histopathology, growth, animal welfare.

Resumo

Danio rerio (Hamilton, 1822) é um dos modelos biológicos mais em voga atualmente. Apesar da sua enorme importância em várias áreas da ciência e biomedicina, o seu cultivo não está devidamente padronizado e é feito com base em observações e em condições utilizadas noutras espécies de aquacultura. Para assegurar resultados reprodutíveis e sólidos, a padronização do cultivo desta espécie tem que ser assegurada e para isso é importante realizarem-se estudos para definir as condições ideais de cultivo. Uma das condições de cultivo não definidas para o peixe-zebra é a presença de nitratos. O nitrato é considerado por muitos como não tóxico dado que os peixes possuem maior resistência a este ião do que aos iões amónia e nitrito, sendo muitas vezes negligenciado. No entanto, em sistemas de recirculação de água (RAS) os nitratos podem atingir valores elevados. O presente estudo tem como objetivo determinar os efeitos da exposição crónica a nitratos nos tecidos do peixe-zebra e no seu desempenho.

Com isto proposto, juvenis de peixe-zebra foram expostos às concentrações de 0, 100, 200 e 400 mg/L NO_3^--N durante 28 dias. Como $NaNO_3$ foi usado como fonte de nitrato, também foram colocados juvenis de peixe-zebra à concentração de 1.7 g/L de NaCl de forma a igualar as concentrações de sais da maior concentração de $NaNO_3$. Também, no sentido de avaliar se a amónia acumulada pode influenciar o desempenho dos peixes em sistema estático, juvenis foram também colocados num sistema de recirculação de água. No final da exposição, os peixes foram eutanasiados, pesados, fixados e processados para análise histológica, utilizando técnicas convencionais. Os efeitos histopatológicos foram determinados utilizando o sistema semi-quantitativo proposto por Bernet et al. (1999) e modificado por Saraiva et al. (2015).

Os resultados obtidos indicam claramente que a exposição crónica de juvenis *de D. rerio* a concentrações de nitratos superiores a 200 mg/L NO_3^--N causou efeitos deletérios no crescimento e estado de saúde. Também foi verificado que as alterações histológicas observadas nas brânquias, pele, rim e intestino foram causadas principalmente pela concentração de nitrato, mas as observadas no fígado foram causadas principalmente pela concentração iónica. Os principais fenómenos histopatológicos observados nas brânquias foram edema, hiperémia, hemorragia, hiperplasia e necrose; na pele foram descamação da epiderme e em alguns casos também derme devido a necrose; no rim foram degeneração vacuolar das células epiteliais dos túbulos renais, hiperémia e hemorragia, depósitos no lúmen dos túbulos renais, alguma atrofia dos túbulos renais e necrose do tecido intersticial hematopoiético; no fígado foram vacuolização e hipertrofia dos hepatócitos, necrose e

depleção das reservas lipídicas; no intestino foram vacuolização e hipertrofia dos enterócitos do intestino posterior, hiperplasia de células caliciformes no intestino anterior e atrofia das vilosidades.

Espera-se que estes resultados contribuam para a standardização das condições de cultivo do peixe-zebra e que sejam tomados em consideração em futuros estudos.

Palavras-chave

Peixe-zebra, nitrato, toxicidade crónica, histopatologia, crescimento, bem-estar animal.

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ABW	Average body weight
DM	Dry-matter
EDTA	Ethylenediaminetetraacetic acid
FBW	Final Body Weight
H&E	Haematoxylin-Eosin
IBW	Initial Body Weight
IC	Index for circulatory disturbances
IP	Index for progressive changes
IR	Index for regressive changes
OI	Organ index
ppt	Parts per thousand
RAS	Recirculating Aquaculture System
SD	Standard deviation
SGR	Specific Growth Rate
SL	Standard Length
TL	Total Length
UV	Ultraviolet
WG	Weight gain

1. Introduction

1.1. Zebrafish taxonomy

According to Integrated Taxonomic Information System (itis.org), zebrafish taxonomic position is the following.

Kingdom: Animalia
Phylum: Chordata
Subphylum: Vertebrata
Superclass: Osteichthyes
Class: Actinopterygii
Subclass: Neopterygii
Infraclass: Teleostei
Superorder: Ostariophysi
Order: Cypriniformes
Infraorder: Cyprinoidea
Family: Cyprinidae
Genus: *Danio* Hamilton, 1822
Species: *Danio rerio* (Hamilton, 1822)

Figure 1 - Zebrafish specimen (Source: http://zfatlas.psu.edu/view.php?s=260&atlas=8).

1.2. Morphologic characteristics

Zebrafish is a freshwater teleost and is characterized by its fusiform and laterally compressed body shape. Has a terminal oblique mouth directed upwards and the lower jaw is more protruded than the upper. The eyes are central and invisible from above (Spence et al., 2008). On both sides of the mouth, zebrafish has thin barbells measuring approximately 1.0mm in length and vertically hanged. Zebrafish features a pattern of five alternating blue-black and silvery-yellow longitudinal stripes along the body and on anal and caudal fins. The blue-black stripes contains two types of pigment cells, melanophores and iridophores and the silvery-yellow stripes contains xantophores and iridophores (Schilling, 2002).

Initially, adults (more than 90 days post-fertilization) have an average Total Length (TL) of 2 to 3 cm and during its life span can reach sizes of 4 to 5 cm, although rarely exceeding 4 cm Standard Length (SL) (Schilling, 2002; Spence et al., 2008). The head measures 10 to 15% of the length of the body and the body depth is usually less than 1 cm and 0.2 to 0.3 cm wide. Also, zebrafish have a complete lateral line from the operculum to the tail fin (Schilling, 2002). Males and females can be distinguished by its general body shape. Males are more slender and darker in colour and females are more rotund and present genital papilla near the anal fin (Schilling, 2002; Snekser et al., 2010).

Early stages of zebrafish larvae are long and narrow, with short and rounded heads that become more pointed as the mouth protrudes. An uninterrupted median fin surrounds the trunk and tail with one small interruption at the urogenital opening. In the unpigmented regions, zebrafish early stages are transparent and its internal features are visible. The transition between larval and juvenile is gradual and starts at about 2 weeks post-fertilization (5 mm). The major changes are the development and formation of the adult pattern of pigment stripes, the formation of the adult fins and the ossification of the skeleton (Schilling, 2002).

1.3. Geographic distribution and habitat

Zebrafish is native from south Asia, in countries like India, Nepal, Bangladesh and Pakistan. Its distribution is centred on the Ganges and Brahmaputra river basins but it is also reported that some specimens were collected in the Indus, Cauvery, Pennar, Godavari and Mahanadi river basins (Spence et al., 2008). This geographic region has a monsoon climate with wide seasonal variation with pronounced rainy and dry seasons that affect the habitat parameters (Lawrence, 2007; Spence et al., 2008).

D. rerio can be found in relatively warm sites, although it has a wide temperature plasticity since its natural range can vary from as low as 6 ºC in winter to over 38 ºC in summer. Zebrafish inhabits in slow-moving waters with generally high transparency, including rice fields and other man-made structures, slower reaches of streams and also in foothill streams (McClure et al., 2006; Spence et al., 2006; Spence et al., 2008). The substratum of its habitat consists on mud, sandy mud, clay, silt, cobble and boulders. These waters inhabited by zebrafish have low salinity, varying between 0 and 0.8 ppt and pH is mostly at 8, but can reach 5.9 (Engeszer et al., 2007; McClure et al., 2006; Spence et al., 2006).

1.4. Feeding habits

The zebrafish is an omnivorous fish and its natural diet is mainly composed by zooplankton and insects (Spence et al., 2008). It's reported, by general observations and gut content analysis, that zebrafish also feeds on a wide variety of animal and vegetal matter such as phytoplankton, filamentous algae and vascular plant material, spores, fish scales, invertebrate eggs, arachnids, detritus, sand and mud. One of the most common constituent of the zebrafish gut contents in the wild is dipteran larvae (Spence et al., 2007; Watts et al., 2012). Adult zebrafish are also known by preying eggs and larvae of its own species (Spence et al., 2008).

1.5. Reproduction

D. rerio is a protogynous species considering that initially all gonads develop as ovaries, which approximately half of the population start to differentiate in males between 5 to 7 weeks post hatching (10 to 15 mm of total length) through an intersexual stage and then developing into normal testes by the third month of development (12 to 17 mm of total length). This differentiation depends on the strain and rearing conditions of the zebrafish (Maack and Segner, 2003; Spence et al., 2008; Takahashi, 1977). Zebrafish's reproductive maturity seems to be size-related rather than age-related since domesticated and wild zebrafish reach reproductive maturity at similar sizes, even having distinct growth rates as stated by Spence et al. (2008).

In nature, zebrafish's spawning is seasonal and males show a territorial behaviour around potential oviposition sites (Spence et al., 2008; Spence and Smith, 2005). Eggs are

released over the substratum without any preparation, males fertilize the eggs and neither sex show parental care (Spence et al., 2008). Eggs are demersal, non-adhesive and measures in diameter approximately 0.7 mm and the hatching takes place in nature between 4 to 7 days (Lawrence, 2007; Spence et al., 2008).

1.6. Zebrafish - Biological research model

Zebrafish has become one of the most used biological research model in many areas of biology such as developmental biology, molecular and cell biology, neurosciences and genetics, among others. Since the first article ever published about this vertebrate back in 1951, zebrafish utilization as a research model has been growing constantly. Since 1996, its utilization had a significant increase reaching a total number of 1929 scientific papers published in 2012, totalizing 8370 from 1951 to 2012 (Kinth et al., 2013).

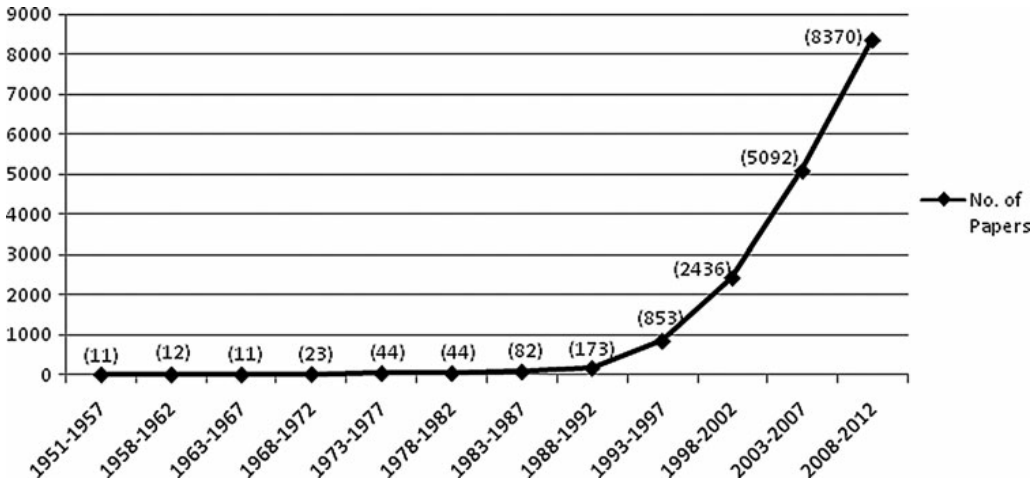

Figure 2 - Annual growth trend of articles from the year 1951 to 2012 utilizing zebrafish as a biological research model (Kinth et al., 2013).

The utilization of *D. rerio* as a research model has many advantages in a large diversity of scientific areas. Considering that it is a small vertebrate, reaching 5 cm in its life span of 2 to 4 years (Schilling, 2002), zebrafish can be kept in large numbers using a small space. This can provide to scientists a considerable amount of experimental conditions, replicates and it is cheaper to rear and maintain than other vertebrates like mice and rats (Lieschke and Currie, 2007).

Humans and zebrafish share a high genome similarity. Approximately 70% of human genes have at least one zebrafish orthologue and 69% of zebrafish genes have at least one human orthologue (Howe et al., 2013) which can provide various insights in human sciences

such as cardiology, infections, nutrition, carcinogenesis, among others areas (Amatruda et al., 2002; Lieschke and Currie, 2007) conceding a better knowing in human physiology and diseases.

Zebrafish can produce nearly 200 eggs per week in favourable conditions and a fertilized egg takes only 3 to 4 months to develop into a reproductive mature individual (Dahm and Geisler, 2006; Maack and Segner, 2003). This short time of development and this high amount of offspring can provide an ample supply of embryos to study all stages of development and turns the renewal of stocks easier. Embryos and early development individuals are nearly transparent allowing easy manipulation of eggs and permitting exams of the development of internal structures in different stages (Kimmel et al., 1995; Schilling, 2002).

1.7. Zebrafish optimal physicochemical parameters in the laboratory

Most of zebrafish housing systems are Recirculating Aquaculture Systems (RAS). This type of systems highly reduces the quantity of water used and the space requirements are lower comparing to flow-through systems (Lawrence and Mason, 2012).

The advised and commonly used photoperiod to rear zebrafish is 14 hours of light and 10 hours of dark, intending to simulate optimal natural conditions (Brand et al., 2002; Matthews et al., 2002; White, 2005). Relatively to temperature there is not yet a consensus. Many authors have recommended various temperature intervals although the temperature recommended by Westerfield (2000) of 28.5 ºC is the most used and cited (Matthews et al., 2002; Reed and Jennings, 2010).

The optimal pH levels for zebrafish growing and reproductive performances has not yet been studied (Lawrence, 2007), although there are some suggestions and recommendations. In nature pH levels can vary between 5.9 and 8.2 (Engeszer et al., 2007; McClure et al., 2006; Spence et al., 2006) and according to Lawrence (2007) most zebrafish facilities aim for keeping pH levels between 7.0 and 8.0. The recommendation of Brand et al. (2002) is to maintain pH levels between 6.8 and 7.5, once levels lower than 6 may cause toxic effects and higher than 8 may prevent the growth of denitrifying bacteria. To adjust pH it's suggested to add carefully bicarbonate or HCl respectively.

Small and tropical fishes like zebrafish have large requirements of dissolved oxygen in water because of their high metabolic rates. In zebrafish facilities it is suggested to

maintain the dissolved oxygen levels at or just under saturation, this is approximately 7.8 mg/L at 28.0 ºC (Lawrence (2007). In terms of salinity and hardness it is suggested a general range of 0.25 to 0.75 ppt and 100 to 200 mg/L $CaCO_3$, respectively (Lawrence, 2007).

Freshwater fishes are constantly excreting ammonia into the water across the gills. The decomposition of faeces, presence of dead fish and uneaten food also results in ammonia increase in the system. Nitrogenous wastes affect the water quality and may damage fish health (Lawrence, 2007; Reed and Jennings, 2010). There are no tested recommended values to set ammonia (NH_3 / NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) levels in zebrafish, although Buttner et al. (1993) states that levels higher than 0.02 ppm of ammonia are problematic and also recommends nitrite levels below 1.0 ppm. According to Lawrence (2007) larval zebrafish can tolerate nitrite levels up to 2.0 ppm and doesn't seem to be affected by chronic nitrate levels up to 100 ppm.

1.8. Nitrate in Recirculating Aquaculture Systems (RAS)

Protein is a key ingredient of fish feed, is the major component of the fish muscle and is the source of nitrogen excretion. When the protein is metabolized, its end products are inorganic nitrogen (ammonium ion NH_4^+ in fresh water and ammonia NH3 in sea water), CO_2 and water (Evans et al., 2005; Wilkie, 1997). Both forms of inorganic nitrogen are excreted over the gills and the form which is released is unimportant since both are in equilibrium in water. A small part of the ammonia is also released in the form of urea in the urine (Lekang, 2013b).

Nitrogenous wastes may cause problems to fish health so they must be removed from the system. Most of recirculating aquaculture systems uses biological filters to reduce the presence of nitrogenous wastes and therefore avoid fish health issues. These biological filters are constituted by bacteria that create a biofilm and oxidize ammonium to nitrite and nitrate. The biological removal of ammonium of a system is accomplished by three processes. The first two are known as nitrification and occur simultaneously carried by nitrifying aerobic bacteria: transfer of NH_4^+ to NO_2^- (ammonium ion to nitrite) and transfer of NO_2^- to NO_3^- (nitrite to nitrate). The third process, transfer of NO_3^- to N_2 (nitrate to molecular nitrogen), is known as denitrification and is carried by denitrifying anaerobic bacteria. This last process is often neglected since fish have a higher resistance to nitrate than ammonia and nitrites (Lekang, 2013a).

Biofilters require some time to acquire a biofilm in order to be functional. Firstly, ammonia excreted by cultured fish accumulates in the water until *Nitrosomonas* spp. form in the biofilter, decreasing rapidly the ammonium concentration, implying an accumulation of nitrite. Afterwards, *Nitrobacter* spp. starts to establish in the biofilter, consuming nitrites and increasing the concentration of nitrate in the system (Skjølstrup et al., 1997). As the denitrification filter is frequently absent, nitrate accumulates over time, and can reach concentrations as high as 100 to 1000 mg/L NO_3^--N in intensive culture systems (van-Rijn and Ebeling, 2010) as it is illustrated in figure 3.

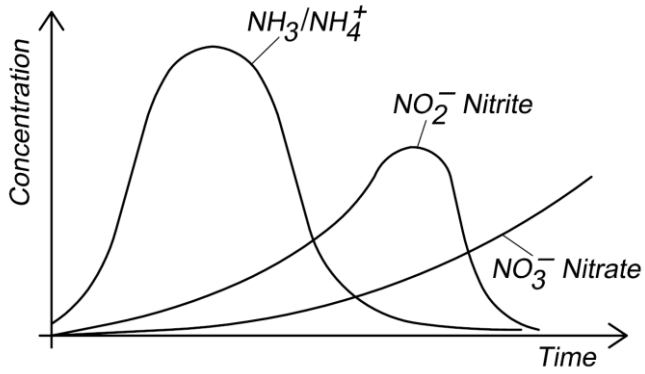

Figure 3 - Evolution of concentration of NH_3/NH_4^+, NO_2^- and NO_3^- over time (Lekang, 2013a).

2. Objectives

The aim of the present study is to evaluate chronic effects of relevant levels of nitrate on the histology of selected organs and on the zootechnical performance of juvenile zebrafish, in order to help defining safe levels of nitrate in zebrafish husbandry.

3. Materials and Methods

3.1. Zebrafish oviposition stimulation

Mature wild-type zebrafish were placed at a ratio of 2 males to 1 female in 4 litres tanks with aeration, subtract (marbles), subjected to a photoperiod of 14 hours and at a temperature of 28 $\pm$ 1^{o}C. For the collection of eggs, and at the same time to avoid the predation by parents, a net with a 2mm mesh was placed above the bottom of the tank.

About two hours after initiation of the light cycle, eggs were collected and placed in round bottom flasks of 2 litres under the same conditions used previously until hatching start. Dead embryos were removed daily to avoid water quality deterioration.

3.2. Larvae - Maintenance and feeding

The larvae were kept in 10 litres plastic tanks in a recirculated water system (RAS). The number of larvae per litre varied according to larvae size to prevent growth problems due to lack of space. The tanks were cleaned daily to avoid water quality deterioration. The larvae were maintained at 28 $\pm$ 1^{o}C and to a 14 hours photoperiod.

After the absorption of the yolk sac, larvae were fed twice a day till the 30th day post-hatching with live *Artemia* spp. nauplii. Additionally, from 23th day post-hatching dry food with granulation between 200 and 400 μm was added as feed (Appendix I).

3.3. Decapsulation of *Artemia* spp. cysts

The decapsulation of *Artemia* spp. cysts was done based on the method proposed by Sorgeloos et al. (1977).The cysts were hydrated in sea water for approximately 90 minutes and then collected by filtration and placed in 100 mL of a buffer solution (6 mL NaOH 40% + 94 mL sea water). Then, 200 mL odourless commercial bleach (refrigerated) was added and, the cysts were maintained in suspension by agitation for 2 to 4 minutes. After collected by filtration, cysts were washed abundantly with tap water and, placed in a 0.6% acetic acid solution for 1 minute under agitation. Finally the cysts were collected by filtration and again washed abundantly with tap water. Thus decapsulated, cysts were stored at 4^{o}C in a 15% NaCl solution until later use, whether for hatching or as providing fresh cysts.

3.4. Obtaining *Artemia* nauplii

The incubation of decapsulated cysts was made in a cylinder conical containers with synthetic sea water under conditions of continuous lighting, intense aeration from the bottom and at a temperature of 20 $\pm$ 1 ^{o}C for 24 hours. After hatching, the nauplii were collected by filtration with a net and washed with system water prior to be used as food.

The synthetic sea water used in the incubation of the decapsulated cysts was obtained by dissolving, at room temperature, 175 g of commercial sea salt in 5 litters of tap water under agitation.

3.5. Toxicological testing - Concentrations and disposition of tanks

It is aimed to evaluate the effects of several concentrations of nitrate in zebrafish juveniles in terms of survival, growth and histology. For this proposed, triplicate randomly selected groups of 10 juveniles (30 days post-hatching) were exposed to 0, 100, 200 or 400 mg/L NO_3^--N for 28 days. A triplicate group were exposed to 1.7 g/L NaCl to match the ionic concentration of the highest nitrate concentration (400 mg/L NO_3^--N) in order to try to distinguish the effects of nitrate from the ones caused by ionic increase. To evaluate whether the accumulated ammonia could influence fish performance an additional triplicate group was placed directly in the recirculating water system.

The nitrate concentrations were obtained from the dilution of a stock solution with a concentration of 400 g/L $NaNO_3$ and the NaCl concentration from a stock solution with a concentration of 175 g/L. The tanks (Fig. 4) were the same ones used in Learmonth and Carvalho (2015) study and were arranged randomly and changed position daily in a recirculation water system (Fig. 5) similar to the one described by Charlon and Bergot (1984).

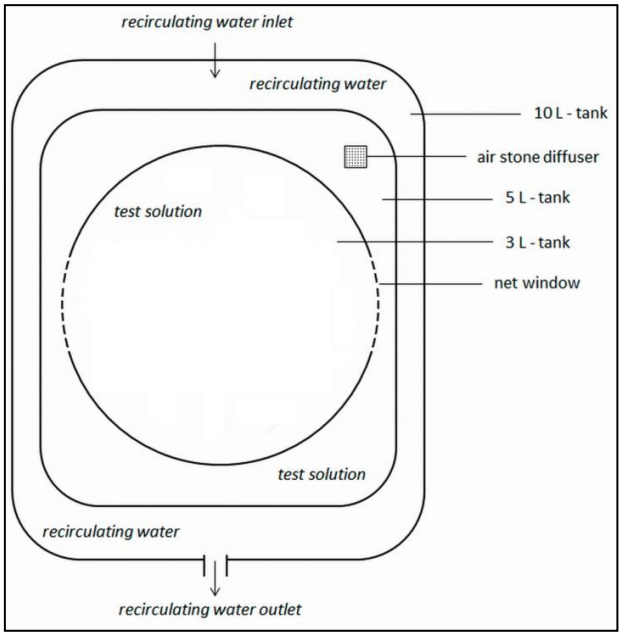

Figure 4 - Schematic representation of an experimental unit used in the chronic bioassay. Modified from Learmonth and Carvalho (2015).

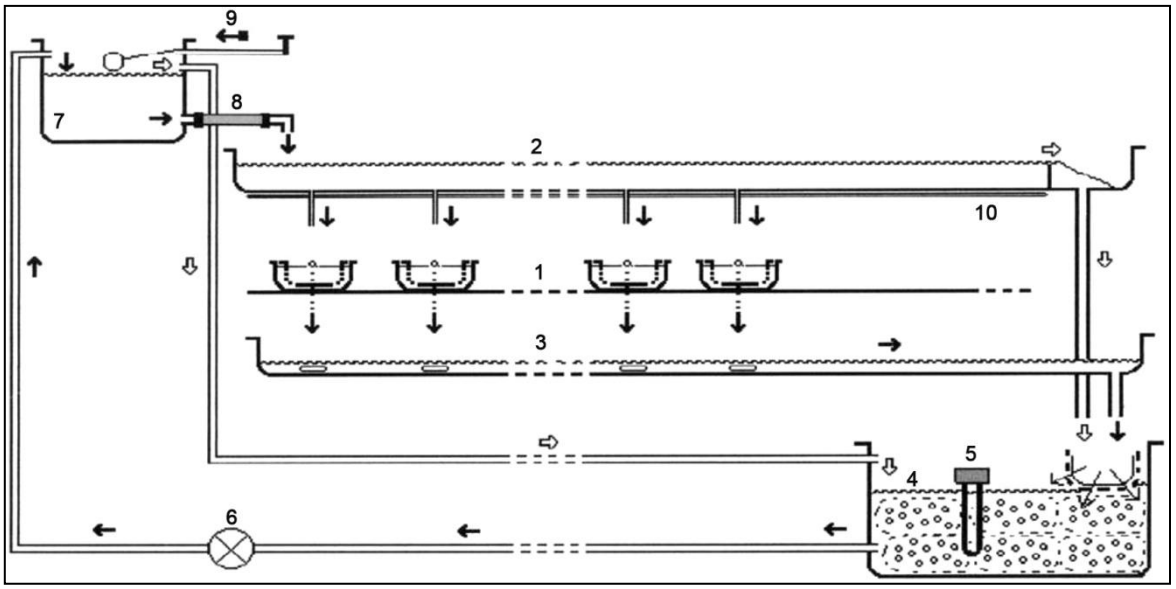

Figure 5 - Charlon and Bergot (1984) recirculation water system scheme. 1 - Experimental units; 2 - Water supply gutter; 3 - Water collecting gutter; 4 - Lower water reservoir with a biological filter; 5 - Heating water device; 6 - Water pump; 7 - Upper water reservoir with a security device; 8 - UV lamp; 9 - Water entrance into the security device; 10 - Fluorescent lamps.

3.6. Feeding and tank maintenance

During the experiment, the fish were maintained at 28 ± 1°C and exposed to 14 hours photoperiod. The physico-chemical water parameters (pH, ammonia, nitrite and nitrate) were recorded using a commercial kit (SERA GmbH) and pH was maintained between 7.8 and 8.3 (8.1 ± 0.2). Ammonia (NH_3) was always kept below 0.03 ppm and nitrite ions (NO_2^-) and nitrate ions (NO_3^-) were always kept at 0mg/L except for the tested concentrations. The fish were fed a formulated feed (Appendix I) with a granulation between 400 and 600 μm, at a daily ration of 3% body weight. The tanks were changed and cleaned every day and new medium was provided to the closed tanks system.

3.7. Sampling

Daily dead and moribund fish were removed. At the end of the exposure time (28 days), 5 fish were randomly taken from each tank. Apart dead fish, all changes in fish (behaviour, shape, colour, etc.) were recorded, and all surviving fish were euthanized using phenoxyethanol anaesthetic overdose and photographed. The total fish weight per tank was recorded weekly and the Weight Gain (WG) and Specific Growth Rate (SGR) were calculated as follow:

$$WG = [(FBW\ (mg) - IBW\ (mg)) / ABW / 1000] / No.\ of\ Days$$

$$SGR = [(\ln(FBW) - \ln(IBW)) / No.\ of\ Days] * 100$$

$$ABW = (IBW + FBW)/2$$

Where IBW = Initial Body Weight; FBW = Final Body Weight; ABW = Average Body Weight

3.8. Histological technique, sectioning and staining

Fixation was done in Modified Davidson's Fixative (Johnson et al., 2009), where water was changed by saline, in a proportion of 1:10 (piece volume : fixative volume) for 48 hours, then changed to 70% ethanol for 24 hours, later to EDTA decalcifying solution (Roberts, 2012a) for 24 hours and finally kept in 70% ethanol until subjected to histological technique (Appendix II).

Four 5 μm thickness sagittal sections were made per fish. The sections were stained using Haematoxylin-Eosin (H&E) staining (Appendix III). The slides were observed under optical microscope and photographed using the Leica Application Suite v.4.6 software. Histological changes were registered according to the semi-quantitative system proposed by Bernet et al. (1999) modified by Saraiva et al. (2015).

3.9. Statistical analysis

Data analysis was carried out using *IBM SPSS Statistics 22* statistic software.

Data from survival rates and weight gain were analysed by one-way analysis of variance (ANOVA). All data were checked for normal distribution and homogeneity of variance and when needed they were transformed. If significant differences were detected ($p < 0.05$) the Tukey multiple range test was used to discriminate means. Histological reaction indices and organ indices, obtained in different nitrate concentrations (control=0/100/200/400), were compared using non-parametric Kruskal-Wallis test, followed by multiple comparisons when significant differences were detected. Histological reaction indices and organ index were also compared between control/continuous flow; 400 mg/L nitrate concentration/NaCl treatment and control/NaCl treatment using non-parametric Mann-Whitney test. Statistical significance was accepted when $p < 0.05$.

4. Results

4.1. Growth and survival

All treatments showed 100% mean survival rate except the ones subjected to a concentration of 400 mg/L NO_3^--N. In the end of the 28th day of exposure, the mean survival rate for the 400 mg/L NO_3^--N tanks was 53% ± 35. The first death occurred in the 13th day of exposure (Fig. 6). Moribund fish were lethargic, or presented brusque swimming followed by lethargy or erratic swimming, and swimming near the surface or in the bottom of the tank. Emaciation, lordosis and superficial lesions on the head, trunk, tail and fins were observed in fish from 400 mg/L NO_3^--N tanks. Growth performance and mean survival rate are summarized in Table 1.

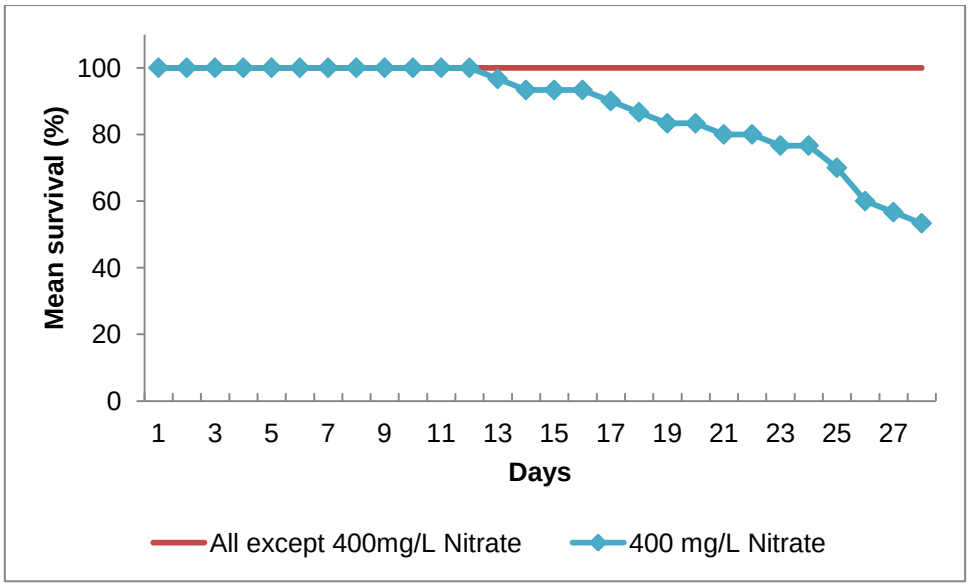

Figure 6 - Mean survival rate during the exposure to the nitrate concentrations, Sodium chloride and in recirculating water.

Although significant differences were detected in weight gain in the 21st day, no significant differences were observed in the end of the experimental period (Fig. 7).

Table 1 - Growth performance and mean survival rate of fish exposed to the experimental concentrations.

	Treatments					
	Control	**100 mg/L NO_3^--N**	**200 mg/L NO_3^--N**	**400 mg/L NO_3^--N**	**1.7 g/L NaCl**	**Continuous flow**
Initial Body Weight (mg)	164.0 ± 18.3^a	163.3 ± 18.9^a	167.3 ± 9.1^a	165.0 ± 8.2^a	164.7 ± 9.0^a	164.7 ± 17.2^a
Final Body Weight (mg)	314.0 ± 8.0^a	305.3 ± 13.0^a	303.7 ± 23.6^a	268.0 ± 53.7*^{a}	316.0 ± 3.6^a	300.0 ± 33.2^a
Weight gain (mg/g ABW^{-1} day^{-1})	22.5 ±2.8^a	21.7 ± 2.4^a	20.6 ± 0.8^a	16.4 ± 4.5*^{a}	22.5 ± 2.0^a	20.6 ± 2.4^a
Specific Growth Rate (%)	2.3 ± 0.3^a	2.2 ± 0.3^a	2.1 ± 0.1^a	1.7 ± 0.5*^{a}	2.3 ± 0.2^a	2.1 ± 0.3^a
Survival Rate (%)	100%^{a}	100%^{a}	100%^{a}	53%±35^b	100%^{a}	100%^{a}

*Results from 2 tanks since in one tank only 2 fishes survived.
Values presented as mean ± SD. Different superscripts in the same line were found to be significantly different ($p < 0.05$) using the one-way analysis of variance and the Tukey's test ($p < 0.05$).

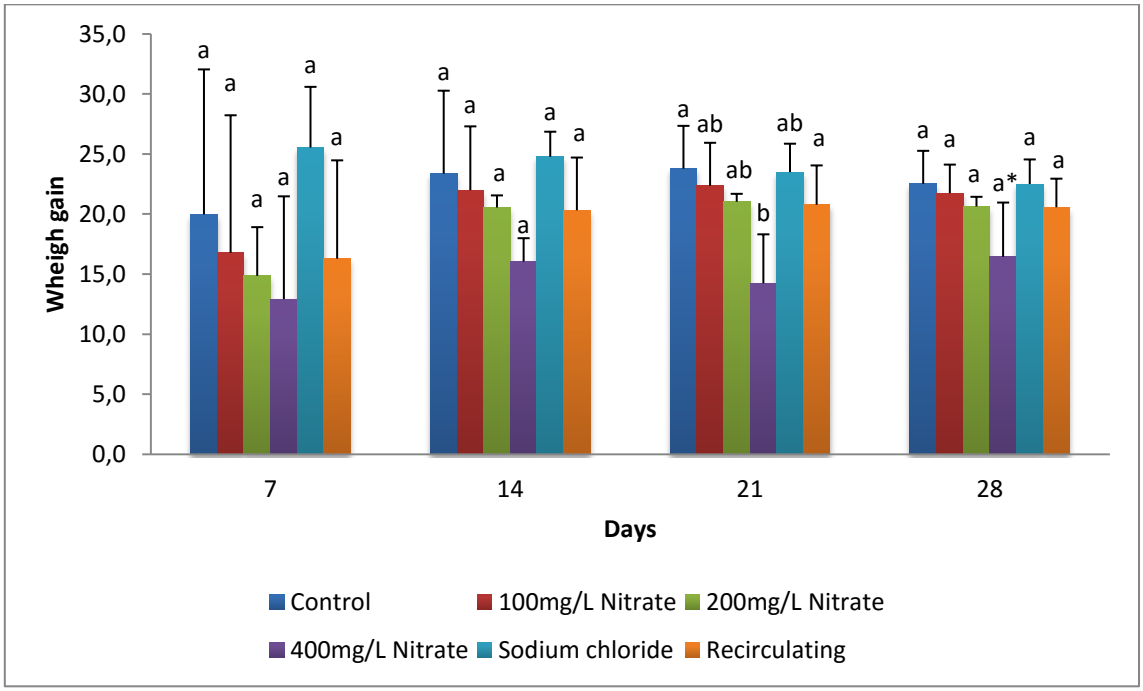

Figure 7 - Weight gain of fish exposed to different concentrations of nitrate, sodium chloride and recirculating water.
Values presented as mean ± SD. Different superscripts in the same day were found to be significantly different ($p < 0.05$) using the one-way analysis of variance and the Tukey's test ($p < 0.05$). * Results from 2 tanks since in one tank only 2 fishes survived.

4.2. Histopathology

4.2.1. Gills

Gill histological reaction index was significantly different in fish reared in different concentrations of NO_3^-. Fish reared in tanks with 200 and 400 mg/L NO_3^--N presented higher values although not significantly different between them (Table 2). The same results were observed when analysed the index for circulatory disturbances (IC) and the index for regressive changes (IR). However the index for progressive changes was higher and significantly different in fish reared in tanks with 400 mg/L NO_3^--N. Significant differences were also observed between fish reared in static (0 mg/L NO_3^--N) and continuous flow and between 400 mg/L NO_3^--N and 1.7g/L NaCl but not between 0 mg/L NO_3^--N and 1.7g/L NaCl.

The normal histology of zebrafish gills is presented in Figure 8A and the histopathological phenomena observed in gills from fish from experimental tanks are presented in Figures 8B to D. Briefly, the main histopathological phenomena observed in gills were oedema, hyperaemia, haemorrhages, epithelial cells hyperplasia and necrosis.

Table 2 - Gills histological reaction indices (IC - index for circulatory disturbances; IR - index for regressive changes; IP - index for progressive changes) and organ index (OI) obtained in different nitrate concentrations (control=0/100/200/400), continuous flow and NaCl treatment.

	Gills indices			
	IC	**IR**	**IP**	**OI**
	Mean (s.d.)	Mean (s.d.)	Mean (s.d.)	Mean (s.d.)
	Range	Range	Range	Range
0 mg/L NO_3^--N (n = 13)	1.5 (2.1) 0-7 **a/α/∗**	0.0 (0.0) 0 **a/α/∗**	0.5 (1.2) 0-4 **a/α/∗**	2.0 (2.5) 0-7 **a/α/∗**
100 mg/L NO_3^--N (n = 14)	1.9 (1.9) 0-6 **ab**	0.1 (0.5) 0-2 **a**	0.3 (0.7) 0-2 **a**	2.4 (1.7) 0-6 **a**
200 mg/L NO_3^--N (n = 12)	3.3 (2.1) 0-6 **ab**	3.3 (3.4) 0-9 **b**	0.3 (1.2) 0-4 **a**	6.9 (5.1) 0-15 **ab**
400 mg/L NO_3^--N (n = 19)	4.0 (2.5) 0-8 **b/B**	4.8 (6.8) 0-30 **b/B**	3.2 (3.6) 0-12 **b/A**	12.0 (8.8) 0-42 **b/B**
Continuous flow (n = 14)	3.2 (1.5) 2-6 #	0.4 (1.6) 0-6 **∗**	4.0 (2.6) 0-8 #	7.6 (3.5) 2-14 #
1.7g/L NaCl (n = 15)	2.1 (1.8) 0-6 **A/α**	1,6 (3.6) 0-12 **A/α**	0.7 (1.2) 0-4 **A/α**	4.4 (4.5) 0-14 **A/α**

For each column small letters indicate statistical comparisons among nitrate treatments (using Kruskall-Wallis test), capital letters, numbers and symbols indicate statistical comparisons between 400 nitrate treatment/NaCl treatment, control/continuous flow and control/NaCl treatment, respectively, using Mann-Whitney test. Similar letters, numbers or symbols indicate no significant differences.

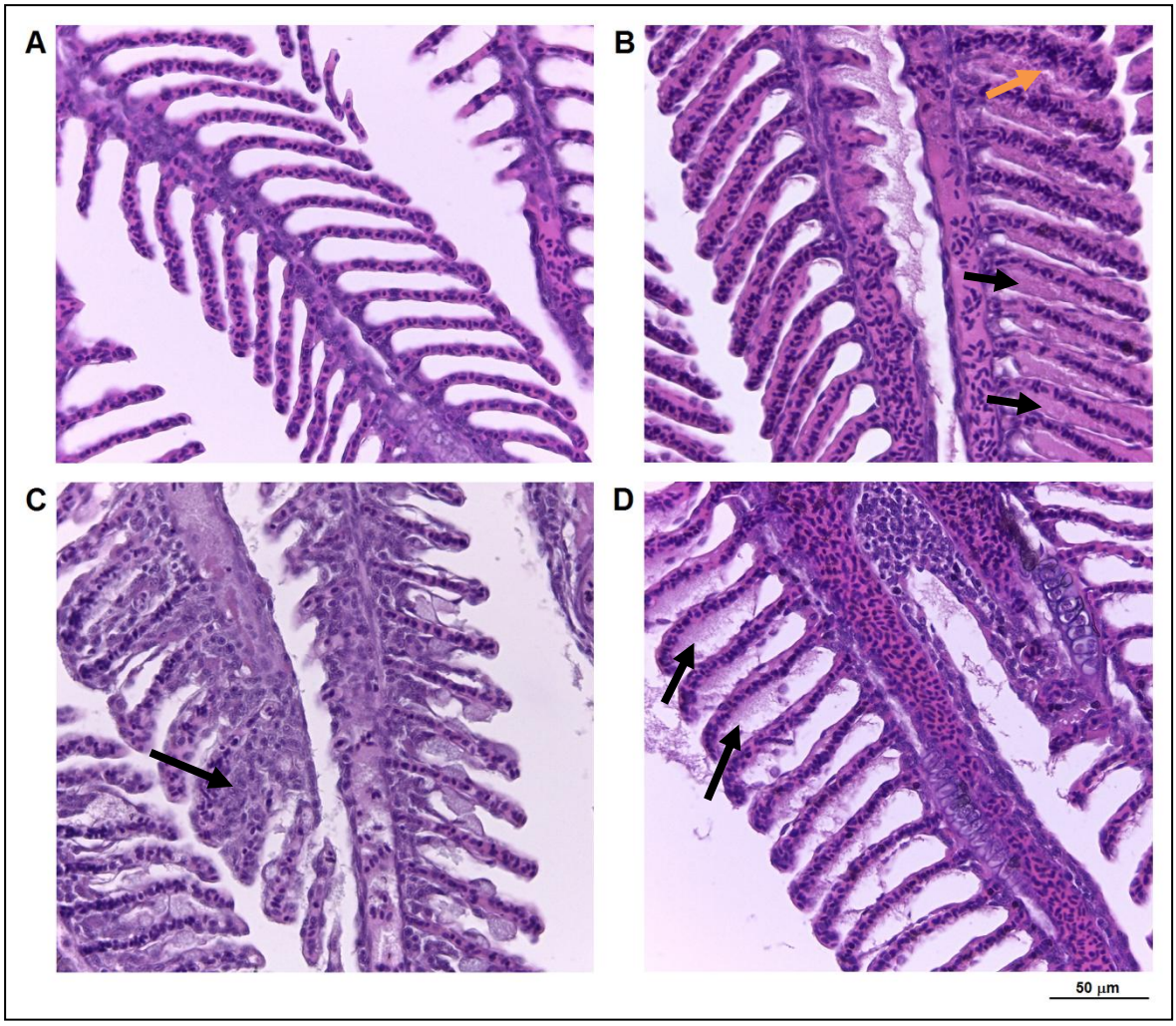

Figure 8 - Representative photos of histological features observed in the gills of zebrafish. A. normal gill; B. gill with oedema (black arrow) and hyperaemia (orange arrow); C. hyperplasia of gills epithelial cells (arrow); D. necrosis of gills epithelial cells (arrows) (H&E).

4.2.2. Skin

Skin histological reaction index was significantly different in fish reared in different concentrations of NO_3^-. Fish reared in tanks with 400 mg/L NO_3^--N presented higher values although not significantly different from the ones from 200 mg/L NO_3^--N (Table 3). The same results were observed when analysed the index for regressive changes (IR). The indices for circulatory and progressive changes were not significantly different in fish reared in tanks with different NO_3^- concentrations. No significant differences were observed between fish reared in static (0 mg/L NO_3^--N) and continuous flow and between 0 mg/L NO_3^--N and 1.7g/L NaCl. However significant differences were observed between 400 mg/L NO_3^--N and 1.7g/L NaCl.

The normal histology of zebrafish skin is presented in Figure 9A and B and the histopathological phenomena observed in skin from fish from experimental tanks are presented in Figure 9C to E. Briefly, the main histopathological phenomena observed in skin were epidermis and in some cases also dermis desquamation due to necrosis.

Table 3 - Skin histological reaction indices (IC - index for circulatory disturbances; IR - index for regressive changes; IP - index for progressive changes) and organ index (OI) obtained in different nitrate concentrations (control=0/100/200/400), continuous flow and NaCl treatment.

	Skin indices			
	IC	**IR**	**IP**	**OI**
	Mean (s.d.)	Mean (s.d.)	Mean (s.d.)	Mean (s.d.)
	Range	Range	Range	Range
0 mg/L NO_3^--N (n = 14)	0.1 (0.4) 0-1 **a/α/∗**	0.0 (0.0) 0 **a/α/∗**	0.0 (0.0) 0 **a/α/∗**	0.1 (0.4) 0-1 **a/α/∗**
100 mg/L NO_3^--N (n = 14)	0.4 (0.9) 0-2 **a**	1.1 (3.0) 0-10 **a**	0.1 (0.5) 0-2 **a**	1.7 (4.0) 0-2 **a**
200 mg/L NO_3^--N (n = 15)	0.3 (0.7) 0-2 **a**	2.7 (3.1) 0-7 **ab**	0.3 (0.7) 0-2 **a**	3.2 (3.3) 0-8 **ab**
400 mg/L NO_3^--N (n = 19)	0.2 (0.7) 0-3 **a/A**	8.7 (7.4) 0-24 **b/B**	1.1 (1.9) 0-6 **a/A**	9.9 (7.4) 0-24 **b/B**
Continuous flow (n = 15)	0.3 (0.7) 0-2 **∗**	0.8 (1.4) 0-3 **∗**	0.0 (0.0) 0 **∗**	1.1 (1.8) 0-5 **∗**
1.7g/L NaCl (n = 15)	0.0 (0.0) 0 **A/α**	0.4 (1.1) 0-3 **A/α**	0.1 (0.5) 0-2 **A/α**	0.5 (1.5) 0-5 **A/α**

For each column small letters indicate statistical comparisons among nitrate treatments (using Kruskall-Wallis test), capital letters, numbers and symbols indicate statistical comparisons between 400 nitrate treatment/NaCl treatment, control/continuous flow and control/NaCl treatment, respectively, using Mann-Whitney test. Similar letters, numbers or symbols indicate no significant differences.

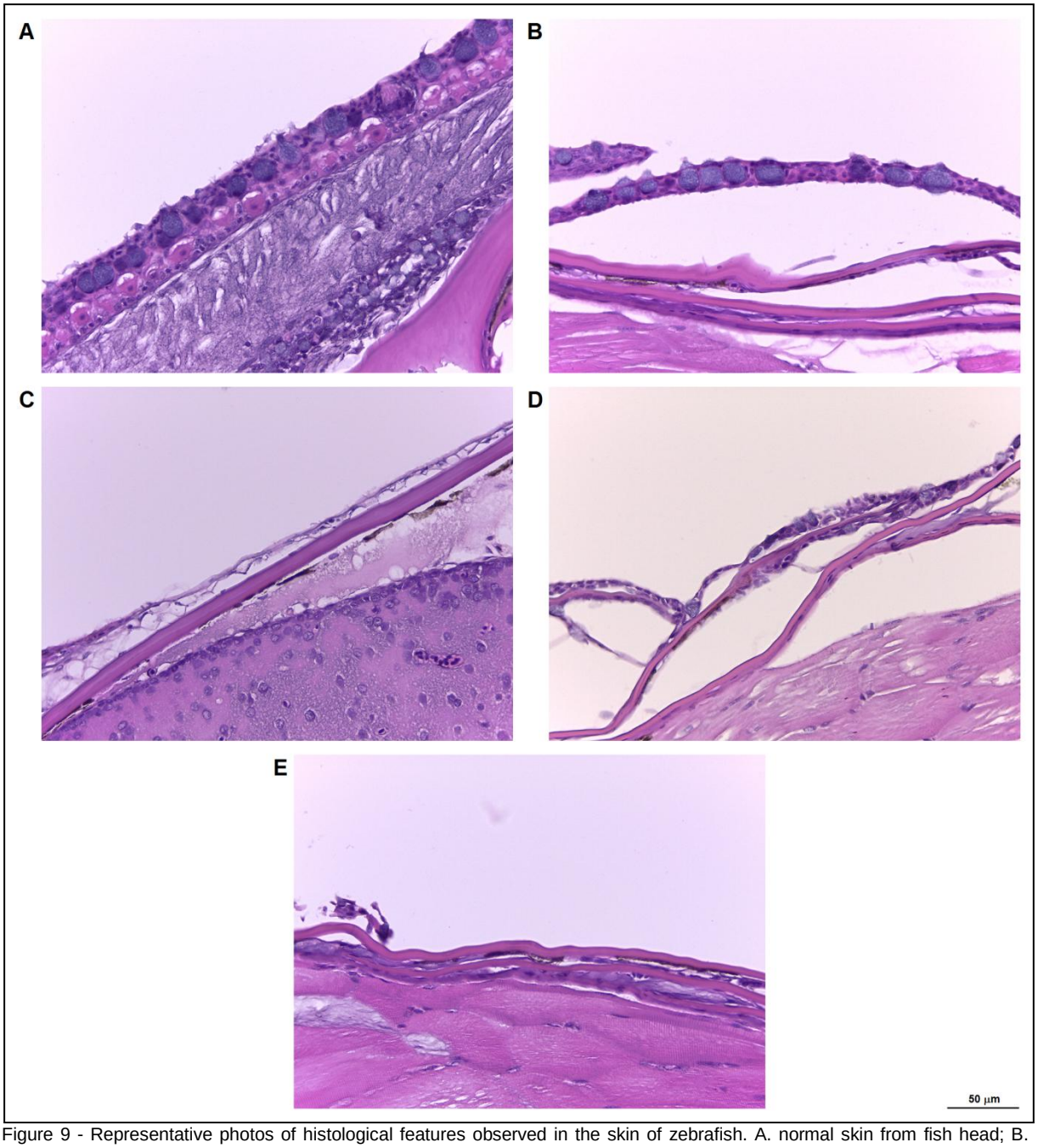

Figure 9 - Representative photos of histological features observed in the skin of zebrafish. A. normal skin from fish head; B. normal skin from fish trunk; C. epidermis absence due to necrosis and desquamation and dermis necrosis from fish head skin; D. epidermis necrosis in skin from fish trunk where still is observed some epithelial cells; E. epidermis and dermis necrosis in skin from fish trunk (H&E).

4.2.3. Kidney

Kidney histological reaction index was significantly different in fish reared in different concentrations of NO_3^-. Fish reared in tanks with 400 mg/L NO_3^--N presented higher values (Table 4). The same results were observed when analysed the index for regressive changes (IR). However the indices for circulatory and progressive changes are not significantly different between fish reared in different concentrations of NO_3^-. No significant histological differences were observed between fish reared in static (0 mg/L NO_3^--N) and continuous flow and between 0 mg/L NO_3^--N and 1.7g/L NaCl. However between 400 mg/L NO_3^--N and 1.7g/L NaCl significant differences were observed.

The normal histology of zebrafish kidney is presented in Figure 10A and the histopathological phenomena observed in kidneys from fish from experimental tanks are presented in Figure 10B to E. Briefly, the main histopathological phenomena observed in kidney were vacuolar degeneration of renal tubules epithelial cells, hyperaemia and haemorrhage, deposits in renal tubules lumen, but only present in fish in the highest nitrate concentration, some renal tubule atrophy and necrosis of the haematopoietic interstitial tissue.

Table 4 - Kidney histological reaction indices (IC - index for circulatory disturbances; IR - index for regressive changes; IP - index for progressive changes) and organ index (OI) obtained in different nitrate concentrations (control=0/100/200/400), continuous flow and NaCl treatment.

	Kidney indices			
	IC	**IR**	**IP**	**OI**
	Mean (s.d.) Range	Mean (s.d.) Range	Mean (s.d.) Range	Mean (s.d.) Range
0 mg/L NO_3^--N (n = 15)	1.9 (1.6) 0-5 **a/α/∗**	0.1 (0.3) 0-1 **a/α/∗**	0.0 (0.0) 0 **a/α/∗**	1.9 (1.8) 0-6 **a/α/∗**
100 mg/L NO_3^--N (n = 14)	1.8 (1.1) 0-4 **a**	0.5 (0.9) 0-2 **a**	0.0 (0.0) 0 **a**	2.4 (1.4) 0-5 **a**
200 mg/L NO_3^--N (n = 15)	2.3 (1.1) 0-4 **a**	1.6 (2.1) 0-6 **a**	0.0 (0.0) 0 **a**	3.9 (2.6) 1-9 **a**
400 mg/L NO_3^--N (n = 19)	1.2 (1.5) 0-5 **a/A**	10.2 (7.3) 0-26 **b/B**	0.4 (1.1) 0-4 **a/A**	11.8 (6.7) 4-26 **b/B**
Continuous flow (n = 15)	1.8 (1.5) 0-4 **∗**	0.0 (0.0) 0 **∗**	0.0 (0.0) 0 **∗**	1.8 (1.5) 0-4 **∗**
1.7g/L NaCl (n = 14)	1.5 (1.3) 0-4 **A/α**	0.9 (2.2) 0-8 **A/α**	0.0 (0.0) 0 **A/α**	2.4 (2.1) 0-8 **A/α**

For each column small letters indicate statistical comparisons among nitrate treatments (using Kruskall-Wallis test), capital letters, numbers and symbols indicate statistical comparisons between 400 nitrate treatment/NaCl treatment, control/continuous flow and control/NaCl treatment, respectively, using Mann-Whitney test. Similar letters, numbers or symbols indicate no significant differences.

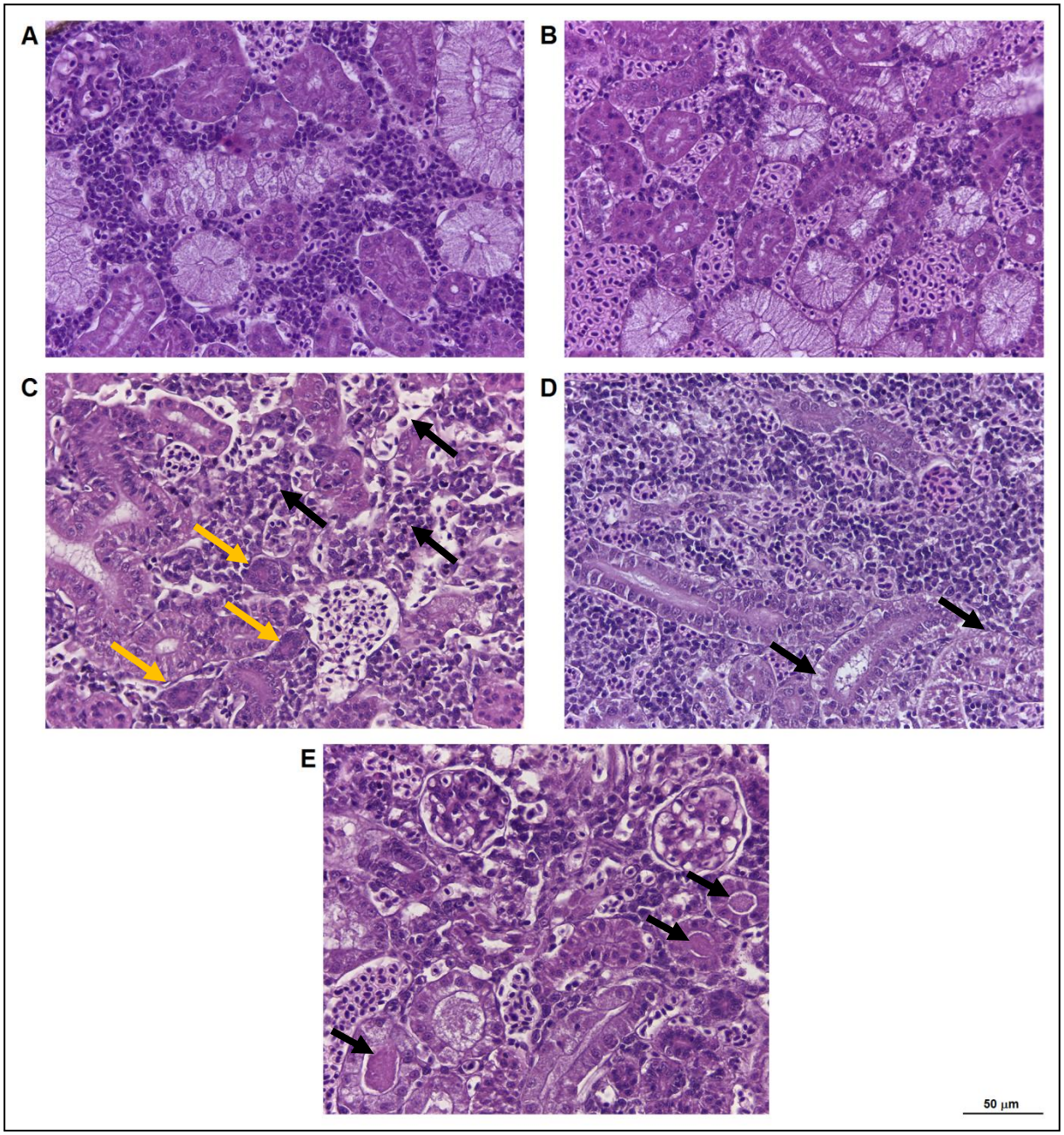

Figure 10 - Representative photos of histological features observed in the kidney of zebrafish. A. normal kidney; B. kidney with hyperaemia and haemorrhage; C. necrosis of the haematopoietic interstitial tissue (black arrows) and renal tubules atrophy (orange arrows); D. vacuolar degeneration of renal tubules epithelial cells (arrows); E. Deposits in renal tubules lumen (arrows) (H&E).

4.2.4. Liver

Liver histological reaction index was significantly different in fish reared in different concentrations of NO_3^-. Although this index gradually increases with increase of tank concentrations of NO_3^- the significance of differences were not very clear (Table 5). The same results were observed when analysed the index for regressive changes (IR). The indexes for circulatory and progressive changes are not significantly different between fish reared in different concentrations of NO_3^-. No significant histological differences were observed between fish reared in static (0 mg/L NO_3^--N) and continuous flow and between fish reared in 400 mg/L NO_3^--N and 1.7g/L NaCl. However significant differences were observed between fish reared at 0 mg/L NO_3^--N and 1.7g/L NaCl.

The normal histology of zebrafish liver is presented in Figure 11A and the histopathological phenomena observed in liver from fish from experimental tanks are presented in Figure 11B to D. Briefly, the main histopathological phenomena observed in liver were hepatocyte vacuolation, hypertrophy, necrosis and depletion of lipid reserves, this last phenomenon only present in moribund fish. Fish from higher ionic concentrations were the most affected.

Table 5 - Liver histological reaction indices (IC - index for circulatory disturbances; IR - index for regressive changes; IP - index for progressive changes) and organ index (OI) obtained in different nitrate concentrations (control=0/100/200/400), continuous flow and NaCl treatment.

	Liver indices			
	IC	**IR**	**IP**	**OI**
	Mean (s.d.)	Mean (s.d.)	Mean (s.d.)	Mean (s.d.)
	Range	Range	Range	Range
0 mg/L NO_3^--N	0.0 (0.0)	0.4 (0.9)	0.1 (0.5)	0.5 (1.4)
(n = 15)	0	0-3	0-2	0-5
	a/α/∗	**a/α/∗**	**a/α/∗**	**a/α/∗**
100 mg/L NO_3^--N	0.0 (0.0)	1.1 (1.5)	0.3 (0.7)	1.4 (2.1)
(n = 14)	0	0-4	0-2	0-6
	a	**ab**	**a**	**ab**
200 mg/L NO_3^--N	0.0 (0.0)	2.9 (2.7)	0.0 (0.0)	2.9 (2.7)
(n = 15)	0	0-9	0	0-9
	a	**b**	**a**	**b**
400 mg/L NO_3^--N	0.0 (0.0)	4.4 (5.8)	0.2 (0.9)	4.6 (6.2)
(n = 19)	0	0-15	0-4	0-16
	a/A	**ab/A**	**a/A**	**ab/A**
Continuous flow	0.0 (0.0)	3.1 (4.4)	0.0 (0.0)	3.1 (4.4)
(n = 15)	0	0-13	0	0-13
	∗	**∗**	**∗**	**∗**
1.7g/L NaCl	0.0 (0.0)	8.0 (3.8)	0.3 (1.0)	8.3 (4.4)
(n = 15)	0	0-16	0-4	0-20
	A/α	**A/β**	**A/α**	**A/β**

For each column small letters indicate statistical comparisons among nitrate treatments (using Kruskall-Wallis test), capital letters, numbers and symbols indicate statistical comparisons between 400 nitrate treatment/NaCl treatment, control/continuous flow and control/NaCl treatment, respectively, using Mann-Whitney test. Similar letters, numbers or symbols indicate no significant differences.

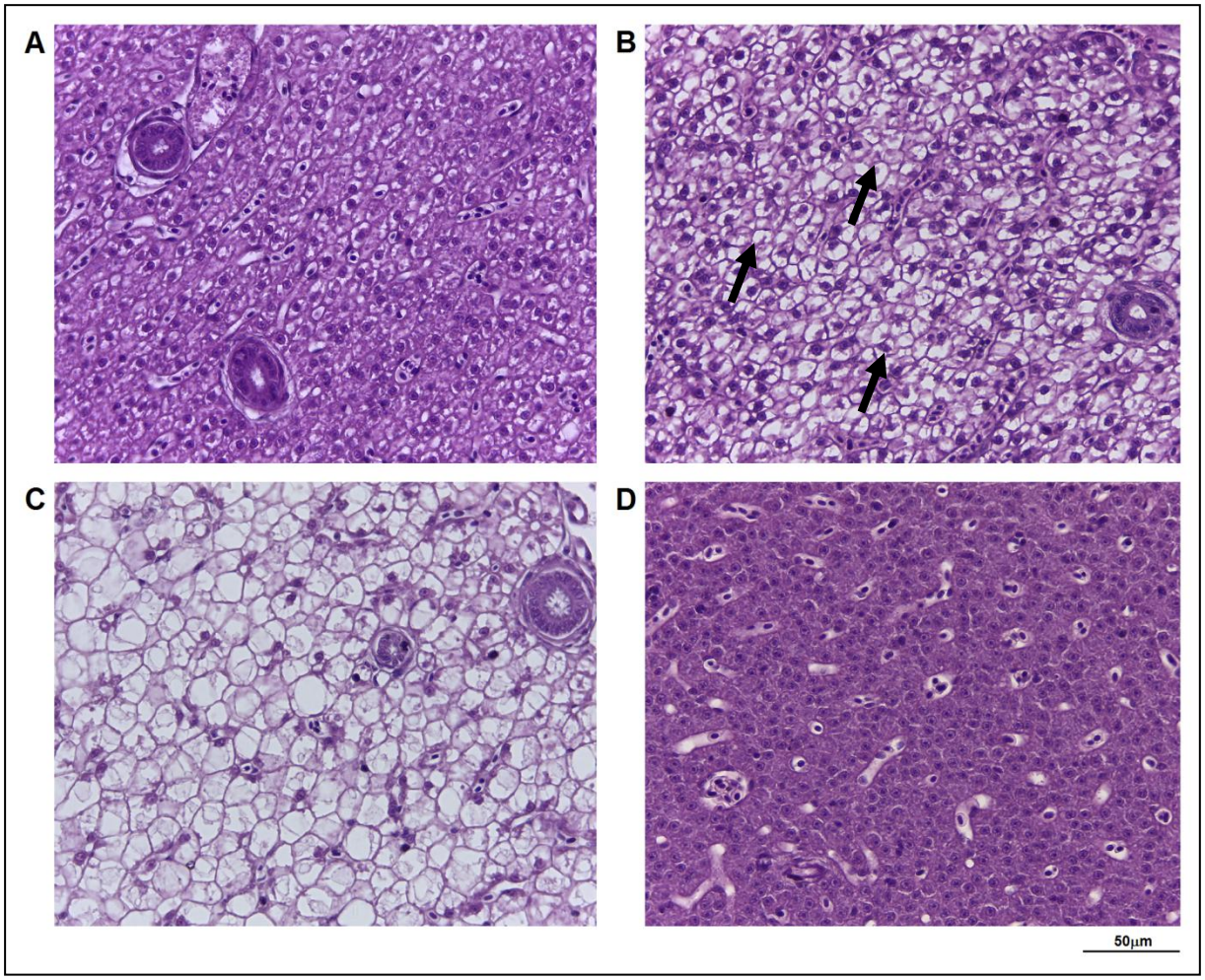

Figure 11 - Representative photos of histological features observed in the liver of zebrafish. A. normal liver; B. hepatocyte vacuolation and slight hypertrophy and necrotic foci (arrows); C. high vacuolation, hypertrophy and necrosis of the hepatocytes; D. liver with very low lipid reserves (H&E).

4.2.5. Intestine

Intestine histological reaction index was significantly different in fish reared in different concentrations of NO_3^-. Fish reared in tanks with 400 mg/L NO_3^--N presented higher values (Table 6). The same results were observed when analysed the index for regressive changes (IR). The significance of differences of the progressive changes index (IP) was not very clear. The index for circulatory changes is not significantly different between fish reared in different concentrations of NO_3^-. No significant histological differences were observed between fish reared in static (0 mg/L NO_3^--N) and continuous flow. No significant differences were observed between 0 mg/L NO_3^--N and 1.7g/L NaCl but differences were significant between 400 mg/L NO_3^--N and 1.7g/L NaCl.

The normal histology of zebrafish intestine is presented in Figure 12A and B and the histopathological phenomena observed in intestine from fish from experimental tanks are presented in Figure 12C to E. Briefly, the main histopathological phenomena observed in intestine were vacuolation and hypertrophy of the enterocytes of the posterior intestine, goblet cells hyperplasia in the anterior intestine and villi atrophy, especially the moribund fish.

Table 6 - Intestine histological reaction indices (IC - index for circulatory disturbances; IR - index for regressive changes; IP - index for progressive changes) and organ index (OI) obtained in different nitrate concentrations (control=0/100/200/400), continuous flow and NaCl treatment.

	Intestine indices			
	IC	**IR**	**IP**	**OI**
	Mean (s.d.)	Mean (s.d.)	Mean (s.d.)	Mean (s.d.)
	Range	Range	Range	Range
0 mg/L NO_3^--N (n = 15)	0.0 (0.0) 0 **a/α/∗**	0.2 (0.6) 0-2 **a/α/∗**	0.9 (1.8) 0-6 **ab/α/∗**	1.1 (2.0) 0-6 **a/α/∗**
100 mg/L NO_3^--N (n = 14)	0.0 (0.0) 0 **a**	0.0 (0.0) 0 **a**	0.4 (1.6) 0-6 **a**	0.4 (1.6) 0-6 **a**
200 mg/L NO_3^--N (n = 15)	0.0 (0.0) 0 **a**	0.1 (0.5) 0-2 **a**	1.1 (2.3) 0-6 **ab**	1.2 (2.2) 0-6 **a**
400 mg/L NO_3^--N (n = 19)	0.0 (0.0) 0 **a/A**	3.5 (4.0) 0-10 **b/B**	2.7 (3.4) 0-12 **b/B**	6.2 (5.4) 0-22 **b/B**
Continuous flow (n = 15)	0.0 (0.0) 0 **∗**	0.4 (0.8) 0-2 **∗**	0.8 (1.7) 0-4 **∗**	1.2 (2.2) 0-6 **∗**
1.7g/L NaCl (n = 15)	0.0 (0.0) 0 **A/α**	0.1 (0.5) 0-2 **A/α**	0.5 (1.4) 0-4 **A/α**	0.7 (1.5) 0-4 **A/α**

For each column small letters indicate statistical comparisons among nitrate treatments (using Kruskall-Wallis test), capital letters, numbers and symbols indicate statistical comparisons between 400 nitrate treatment/NaCl treatment, control/continuous flow and control/NaCl treatment, respectively, using Mann-Whitney test. Similar letters, numbers or symbols indicate no significant differences.

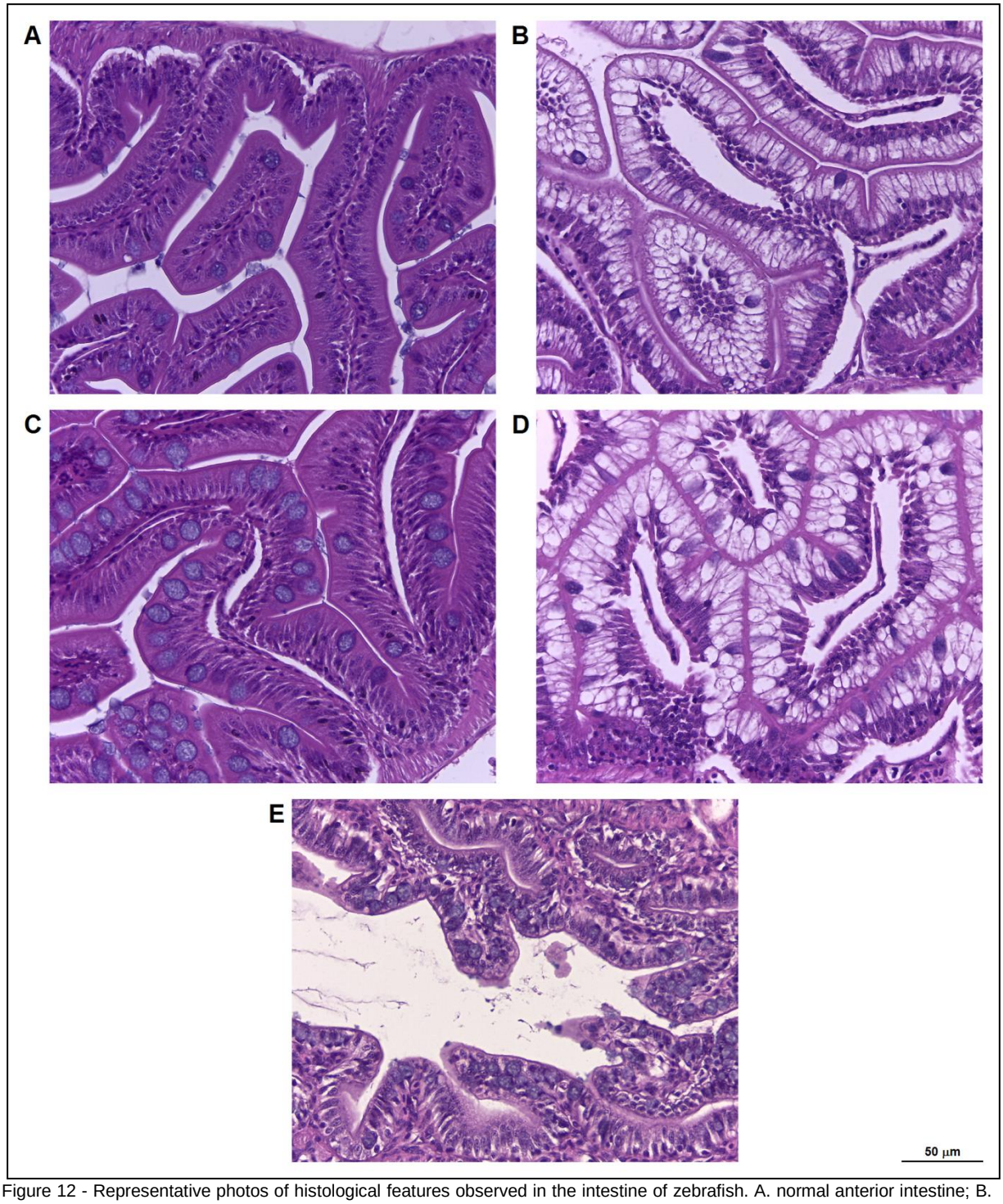

Figure 12 - Representative photos of histological features observed in the intestine of zebrafish. A. normal anterior intestine; B. normal posterior intestine; C. goblet cells hyperplasia; D. vacuolation and hypertrophy of the enterocytes of the posterior intestine; E. villi atrophy (H&E).

4.2.6. Total Indices

Table 7 summarizes the total indices for the circulatory disturbances, regressive changes and progressive changes and the total index (included all organs examined, i.e. gills, kidney, liver, skin and intestine). Total histological reaction index was significantly different in fish reared in different concentrations of NO_3^-. It was observed a gradual increase of these indices related with the increase of NO_3^-. No significant differences were observed between fish reared at 0 and 100 mg/L NO_3^--N, 100 and 200 mg/L NO_3^--N and 200 and 400 mg/L NO_3^--N. It was stated that the main changes observed in fish reared at higher NO_3^- concentrations were caused by regressive and progressive histological changes, been these indices significantly different between fish reared at different concentrations of NO_3^-. No significant differences were observed in total circulatory indices. Significant differences were observed between fish reared in static (0 mg/L NO_3^--N) and continuous flow, between 400 mg/L NO_3^--N and 1.7g/L NaCl and between 0 mg/L NO_3^--N and 1.7g/L NaCl.

Table 7 - Total histological reaction indices (TCI - total circulatory index; TRI - total index for regressive changes; TPI - total progressive index) and total index (TI).

	TCI	TRI	TPI	TI
	Mean (s.d.)	**Mean (s.d.)**	**Mean (s.d.)**	**Mean (s.d.)**
	Range	**Range**	**Range**	**Range**
0 mg/L NO_3^--N	3.3 (3.1)	0.7 (1.0)	1.4 (1.9)	5.4 (4.5)
(n = 15)	0-10	0-3	0-6	0-15
	a/α/∗	**a/α/∗**	**a/α/∗**	**a/α/∗**
100 mg/L NO_3^--N	4.1 (1.7)	3.0 (4.1)	1.1 (1.9)	8.3 (4.7)
(n = 14)	2-7	0-15	0-6	2-19
	a	**ab**	**a**	**ab**
200 mg/L NO_3^--N	5.2 (2.0)	9.9 (5.0)	1.6 (2.3)	16.7 (4.4)
(n = 15)	2-8	1-18	0-6	9-23
	a	**b**	**a**	**bc**
400 mg/L NO_3^--N	5.4 (2.6)	31.6 (15.9)	7.6 (6.2)	44.7 (19.6)
(n = 19)	0-9	9-78	0-24	16-102
	a/B	**c/B**	**b/B**	**c/B**
Continuous flow	5.1 (2.8)	4.7 (4.7)	4.5 (2.7)	14.4 (5.5)
(n = 15)	2-9	0-13	0-10	3-24
	∗	#	#	#
1.7g/L NaCl	3.5 (2.0)	10.9 (3.7)	1.6 (2.7)	16.1 (6.1)
(n = 15)	1-7	6-18	0-10	9-34
	A/α	**A/β**	**A/α**	**A/β**

For each column small letters indicate statistical comparisons among nitrate treatments (using Kruskall-Wallis test), capital letters, numbers and symbols indicate statistical comparisons between 400 nitrate treatment/NaCl treatment, control/continuous flow and control/NaCl treatment, respectively, using Mann-Whitney test. Similar letters, numbers or symbols indicate no significant differences.

5. Discussion

Zebrafish juveniles exposed to higher concentrations of nitrate show less weight gain. This can be verified on the 21st day which significant differences were observed. In the last day of the chronic exposure there were also no significant differences between treatments which may mean that there has been a slight recovery of the fish from the tanks with higher concentration of nitrate or it may be due to the mortality of the weaker and slimmer fishes resulting in an increased fish average weight. This results corroborate the results of Learmonth and Carvalho (2015) that reported high growth problems in zebrafish larval stages exposed for 23 days to 400 mg/L NO_3^--N. This is also observed in other fish species, namely in juvenile turbot *Psetta maxima* (NO_3^--N concentrations above 125 mg/L) and in African catfish *Clarias gariepinus* (NO_3^--N concentrations above 140 mg/L) (Schram et al., 2014; van Bussel et al., 2012). However, *Ictalurus punctatus* growth and feeding activity were not affected by concentrations of 90 mg/L NO_3^--N (Knepp and Arkin, 1973).

Nitrate ion caused mortality in juvenile zebrafish at 400 mg/L NO_3^--N. Levels up to 200 mg/L NO_3^--N did not cause mortality during the experimental period (28 days). This has also been observed in zebrafish larvae exposed for 23 days to the same nitrate concentration (Learmonth and Carvalho, 2015), however in the present study it was also observed that several histopathological phenomena were not significantly different in the gills and skin from fish treated with 200 and 400 mg/L NO_3^--N and this nitrate levels should be avoided in zebrafish RAS. The susceptibility of fish to nitrate is widely variable according to fish species. Mortality in hybrid striped bass *Morone chrysops* × *M. saxatalis* were observed in 200 mg/L NO_3^--N (Hrubec et al., 1996). Salmonid species (*Oncorhynchus mykiss*, *Oncorhynchus tshawytscha* and *Salmo clarki*) exposed for 30 days to nitrate exhibited significant increases in mortality at concentrations from 1.1 to 4.5 mg/L NO_3^--N in developing eggs and early fry and *O. mykiss* juveniles had a slight increase in mortality when exposed to 91 mg/L NO_3^--N (Davidson et al., 2014; Kincheloe et al., 1979). Both adult and growing phases of *Oryzias latipes* chronically exposed to nitrate were lethally affected by a concentration of 100 mg/L NO_3^--N (Shimura et al., 2002; Shimura et al., 2004). Hamlin (2006) reported that NO_3^- concentrations accumulating within RAS may be of concern for Siberian sturgeon *Acipenser baeri* and cited anecdotal evidence that concentrations as low as 90 mg/L NO_3^--N resulted in increased mortality. However a concentration of 358 mg/L NO_3^--N in fathead minnow *Pimephales promelas* for 11 days did not cause mortality (Scott and Crunkilton, 2000). In this study it was stated that sodium chloride concentrations, that equalled the ionic concentration of $NaNO_3$ (1.7g/L NaCl) did not cause mortality but cause

histopathological effects in liver. Learmonth and Carvalho (2015) reported in zebrafish larvae exposed for 23 days at this sodium chloride concentration 70% of mortality. This suggests that later developmental stages of zebrafish are more resistant to ionic concentration/salinity but even so sensitive and negatively affected.

Because of their direct and continuous contact with the environment, the fish gills, which are organs for respiratory gas exchange, osmoregulation, excretion of nitrogenous waste products and an acid-base regulator, are impacted by contaminants (Bhagwant and Elahee, 2002). In this study, it was stated that higher nitrate levels caused more severe effects in gills histology. It was also stated that fish in the continuous flow system presented more histopathological effects than the ones reared in a static system (negative control). This fact may be due to the presence of an abiotic or biotic factor in the water, including the presence of algae detected in the recirculating water system. Gill inflammation, hyperplasia, fusion of the gills, oedema and hypertrophy of epithelial cells were reported for several authors in several aquatic organisms exposed to nitrate (Davidson et al., 2014; Furtado et al., 2014; Hrubec et al., 1996; Kuhn et al., 2010).

Like the gills, the skin present a large superficial area that is in constant and direct contact with potential irritants and it was also stated that higher nitrate levels caused more severe effects in skin histology.

The kidney is an important organ for the internal regulation concerning water and salts, excretion and, in part, to the metabolism of xenobiotics (Bernet et al., 1999). Again it was stated that higher nitrate levels caused more severe effects in kidney histology. Regressive changes like vacuolar degeneration of the renal tubules, necrosis and atrophies are clearly influenced by the ion nitrate since these renal problems were more serious in the highest concentration. That said, the kidney is highly influenced by the presence of nitrate on levels of 400 mg/L. Several authors reported that oedema, dilatation of the renal tubules, mineralization and necrosis of the tubules and renal interstitial fibrosis in fish reared in waters with high nitrate levels (Davidson et al., 2014; Hrubec et al., 1996).

The liver is known by its role in the metabolism and xenobiotics excretion, therefore a primary marker for aquatic pollution (Bernet et al., 1999). The results obtained in this study may indicate that the liver is more susceptible to salinity / sodium ion in the water than to the presence of nitrate. Significant changes were detected on levels above 200 mg/L NO_3^--N, that correspond to a salinity of 0.8 mg/L NaCl, the maximum observed in zebrafish habitat (Learmonth and Carvalho, 2015; Spence et al., 2006). On chronic exposures to nitrate it has been reported a number of liver cells decrease, disruption of cell alignment, infiltration of

lymphocytes, fibrosis, vacuolation and necrotic foci in fishes inhabiting marine or brackish waters (Hrubec et al., 1996; Shimura et al., 2004). Further studies must be carried to a better understanding of the effects of the salinity or of the sodium ion in zebrafish liver and on its performance.

Besides the importance for the absorption of nutrients, intestinal epithelium is also an important site for immunity, osmotic balance, recycling of enzymes and macronutrients (Alvarez-Pellitero, 2011). Despite this fact, pathology of intestines of fish is poorly studied and the major available information is often based in casual observations (Roberts, 2012b). This study reveal that nitrate influence the histological structure of intestine causing vacuolation and hypertrophy of the enterocytes and goblet cells hyperplasia and, mainly in moribund fish, atrophies of the intestinal villi.

Overall levels of exposure to nitrate higher than 200 mg/L causes negative effects on zebrafish juveniles. It is generally accepted that the nitrate toxic action is due to its endogenously conversion into nitrite that reacts with haemoglobin forming methaemoglobin reducing the capacity of carrying oxygen to the tissues (Camargo and Alonso, 2006; Hill, 1999). Nitrate also has the potential to decrease the immune response, to induce haematological and biochemical changes indicative of stress responses and to disrupt endocrine function such as steroidogenesis, which can be problematic to mature zebrafish (Freitag et al., 2015; Guillette and Edwards, 2005; Hamlin et al., 2008; Hrubec et al., 1996; Jannat et al., 2014). Apart from the influence of the ionic concentrations in the liver, other organs were not significantly affected by salinity and did not contributed negatively to its overall performance and condition. This may prove quite advantageous in zebrafish rearing given that salinity increases the lifetime of live food (Jomori et al., 2013) and can be used as a prophylactic treatment for pathogens (Noga, 2011), however, as stated earlier, further studies must be carried on this matter.

6. Conclusion

The results obtained clearly indicate that histological changes observed in gills, skin, kidney and intestine were mainly caused by nitrate concentration but the ones observed in liver were caused mainly by ionic concentration.

This chronic test can provide several insights in what can occur in nature and in RAS, where the concentrations of this ion can reach values between 100 and 1000 mg/L. Therefore, it should be taken in consideration that the exposure to higher concentrations than 200 mg/L NO_3^--N induces sub-lethal and lethal effects in zebrafish juveniles. In conclusion, nitrate constitutes a risk to growth, survival and welfare of zebrafish and it is highly recommended to keep nitrate levels below 200 mg/L in zebrafish rearing.

Despite the importance of zebrafish as a widely used biological research model, its rearing is not standardised and many other parameters must be studied to ensure optimal rearing conditions and to prevent possible bias of results in future experiments.

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8. Appendixes

8.1. Appendix I - Ingredients and composition of used feed

Used feed	
Ingredients (*g / kg diet in dry matter*)	
Fish meal [1]	596.8
CPSP [2]	30
Cod liver oil	38.5
Dicalcium phosphate	25.9
Mineral premix [3]	10
Vitamins premix [4]	5
Choline chloride (50%)	5
Binder [5]	10
Pregelatinized maize starch [6]	278.8
Composition	
Dry matter (dm, %)	94.3
Crude protein (% dm)	45.7
Total lipid (% dm)	8.8
Ash (% dm)	14.6
Gross energy (KJ/g dm)	18.7

[1] Pesquera Centinela, Steam Dried LT, Chile (CP: 71.4%; CL 9.3%). Sorgal, S.A. Ovar, Portugal.
[2] Soluble fish protein concentrate, Sopropêche, France (CP: 80.4% DM; GL: 19.7% DM).
[3] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).
[4] Vitamins (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[5] Aquacube. Agil, UK.
[6] C-Gel Instant – 12016, Cerestar, Mechelen, Belgium.

8.2. Appendix II - Histological technique

Histological technique	
Fixation Bouin*, buffered formalin 10% or Davidson's fixative modified. *washing in alcohol 70% until colourless.	48 hours
Dehydration Alcohol 70% Alcohol 95% Absolute alcohol I Absolute alcohol II Absolute alcohol + salicylate (1:1) Methyl salicylate	 4 - 8 hours 4 hours 2 hours 3 hours 3 hours Over night
Impregnation Salicylate + Paraffin (1:1) Paraffin I Paraffin II	 For 3 hours 2 hours 2 hours
Inclusion	
Sectioning	
Collage	
Drying in oven at 40ºC	
Deparaffinisation Xylene I Xylene II Alcohol + Xylene (1:1)	 2 minutes 2 minutes 2 minutes
Rehydration Absolute alcohol Alcohol 95% Alcohol 80% Alcohol 70% Washing in tap water	 2 minutes 2 minutes 2 minutes 2 minutes
Staining	
Dehydration Alcohol 95% Alcohol 95% II Absolute alcohol Absolute alcohol + Xylenne (1:1)	 2 minutes 2 minutes 2 minutes 3 minutes
Diaphanisation Xylene	2 minutes
Montage	

8.3. Appendix III - Haematoxylin and Eosin staining

Haematoxylin and Eosin Staining
After deparaffinise and hydrate the sections:
1 - Immerse the sections in haematoxylin (Staining I) for 6 minutes.
2 - Wash in tap water for 5 minutes.
3 - Immerse in hydrochloric alcohol 1% for 3 seconds
4 - Quick wash
5 - Immerse in saturated lithium carbonate solution for 3 seconds.
6 - Quick wash.
7 - Immerse in eosin (Staining II) for 3 minutes.
8 - Wash in water.
9 - Dehydrate, diaphanise and mount.