UNIVERSITY OF NAPLES FEDERICO II

DEPARTMENT OF BIOLOGY

PhD Thesis in Biology
(XXXIII Cycle)

Neurobehavioural, histological, physiological and gene expression analyses in zebrafish exposed to Aluminium

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Anche se il timore avrà più argomenti,

Tu scegli sempre la Speranza.

(Seneca)

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1. Introduction

1.2 Aluminium

Aluminium (Al) is a ubiquitous element, naturally occurring in the soil, air, and water. Its presence in the environment has been increasing due to anthropogenic activities (Pereira et al., 2013) and the consequent pollution, especially in water bodies. According to the World Health Organization (WHO), the concentration of Al can vary significantly in natural waters, depending on various physicochemical factors, as mineralogical and pH variations. Normally, dissolved Al concentrations in water range from 0.001 to 0.05 mg/L when the pH is near-neutral values (WHO, 1997, 2013). However, some studies showed that its concentration can vary from 0.014 mg/L in groundwaters to 2.57mg/L in surface waters (Agarwal et al., 2016) and even rise to 50 mg/L due to acid rains (ATSDR, 2008) that, changing the acidity of the water, facilitate the solubilisation of the inorganic Al (Lawrence et al., 2007; Zhang et al., 2016). As a result of its increase in the environment, the Al has been the subject of several studies in the last few years, demonstrating its toxicity for different organisms for which it can alter the survival and growth (Cardwell et al., 2018; Gensemer et al., 2018), but also lead to abnormalities in reproduction (Yokel, 2020) or histopathological alterations affecting different organs (Jagoe and Haines, 1997; Poléo et al., 2017). Although it is so widespread, it has no physiological role in metabolic processes (Exley and House, 2011); but rather Al accumulation in mammalian tissues was associated with various pathologic effects: pulmonary lesions (Kongerud and Søyseth, 2014), bone abnormalities (Klein, 2019), and neurologic disorders (Colomina and Peris-Sampedro, 2017; Morris et al., 2017). Indeed, Al can rapidly enter the brain, extracellular fluid and the cerebrospinal fluid (Krewski et al., 2007) and its accumulation in the brain has been associated with neurodegenerative diseases such Alzheimer's disease (AD), Parkinson's disease (PD), amyotropic lateral sclerosis (SLA) (Arain et al., 2015; Bjorklund et al., 2018; Jones et al., 2017; Kawahara and Kato-Negishi, 2011). Furthermore, Al exposure can promote oxidative stress in the nervous tissue, inducing neurodegeneration, neuronal necrosis and dysneurogenesis, which constitute the basis for the neurological diseases associated with Al toxicity (Bondy, 2016; Exley, 2006). Despite all these evidences, its mechanism of toxicity has not been elucidated. This has made it an interesting element to study in order to better understand its ecological impact, due to its growing diffusion, and the possible implications on human health.

1.3 Model organism: Danio rerio

Danio rerio

Danio rerio, is a small freshwater vertebrate, commonly called zebrafish due to the streaks present along the body. It is a few centimetres long and it is native to southeast Asia It has a slight sexual dimorphism as the females appear bigger than the males, that have a more tapered body (Spence et al., 2008). The use of zebrafish as a model organism began in the 1960s (Spitsbergen et al., 2003), and it was chosen as a model organism in this study for its characteristics that made it perfectly suited to the objectives. Zebrafish is suitable for screening of ecotoxicological effects induced by environmental pollutants both as an adult organism and in the early stages of its embryonic development (Favorito et al., 2011; Lanzarin et al., 2019; Monaco et al., 2016, 2017a). Its eggs, which develop outside the mother's, are produced regularly and in large numbers. They are transparent and this allows you to easily follow all of its embryonic development and to evaluate all the possible repercussions induced by a pollutant at the level of organogenesis (Yang et al., 2009).

In recent years, zebrafish has also consolidated itself as an excellent model organism for neurobehavioral studies, since it shows neuropathological behaviour, quantifiable and connectable to those seen in other mammalians (Best and Alderton, 2008; Guo, 2004; Orger and de Polavieja, 2017).

Ten years ago, zebrafish were identified as an up and coming model for genetic disorders and developmental biology in humans (Zon, 1999). Since this time, the zebrafish genome has been fully sequenced and many genes of high mammalian homology have been identified (Tierney et al., 2011).

Danio rerio during embryonic development

Finally, it has become an important animal model for exploring neurodegenerative diseases because its nervous system is simple, yet it shares functional and anatomical similarities with the human nervous system (Babin et al., 2014).

2. Aim of study

The aim of the study was to evaluate the Al toxic effects in order to better understand its mechanism of action. Research was conducted both on adult organisms and during the embryonic stage.

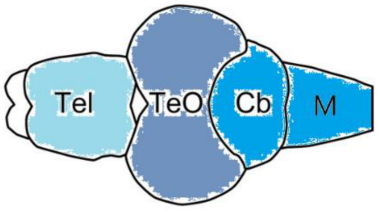
Embryos were exposed from 6 hours post fertilization (hpf) for 72 hours at increasing Al concentrations of 1.40, 2.80 and 5.60 mg/L, respectively, using as a source $AlCl_3 \cdot 6H_2O$ dissolved in water, at concentrations of 50, 100 and 200 µM respectively.

At the end of treatments, it was assessed:

- Morphology
- Time of Hatching
- Motility by DanioVision instrument
- Apoptosis
- Oxidative state
- Gene expression of neuronal development' markers

Adult organisms were exposed to concentrations of 11 mg/L of Al, using as source 400µM (0.1 g/L) of $AlCl_3 \cdot 6H_2O$ and the effect were analysed after 10, 15 and 20 days of exposure.

Analyses were conducted for the evaluation of swimming activity, quantized using the ToxTrac: software Organism video Tracking Application (Umeå University, Linneaus Väg, SE-901 87 Umeå, Sweden Ver.2.84) and the behavioural activities, evaluating the interaction between individual-individual and individual-environment. In light of these analyses, particular attention was paid to the main organs involved in these activities, as brain and musculature. Furthermore, the neurotoxic role of Al was investigated, with particular interest in understanding the correlation between exposure to this metal and neurodegeneration. At the different times of exposure, the effects of Al were evaluated on brain, analysing:



- Histology
- Oxidative state
- Al concentration
- Myelin integrity
- Neurodegeneration
- Immunohistochemistry revelation of the GFAP, glial marker
- Gene expression of markers involved in Parkinsonism

At the different times of exposure, the effects of Al were evaluated on musculature, analysing:

- Histology
- Oxidative state

Finally, at the same exposure times, the alterations induced by Al on the gills, the first organ to come into contact with the metal once dissolved in water, were also analysed. In this regard, it was assessed:

- Histology
- Oxidative state

Overall, these concentrations were chosen to simulate conditions found in polluted surface waters (Agarwal et al., 2016, ATSDR, 2008), taking also into account of the Al-dose effect on survival of these organisms, calculated and published previously (Monaco et al., 2017b).

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Effects of aluminium and cadmium on hatching and swimming ability in developing zebrafish

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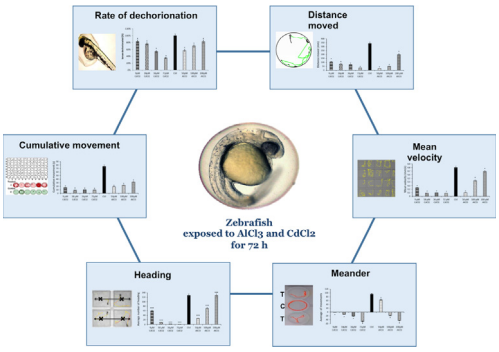
HIGHLIGHTS

- Aluminium and cadmium are toxic metals for developing zebrafish.
- Exposure to $CdCl_2$ induces delay in hatching and in swimming dose-dependent.
- Exposure to $AlCl_3$ increases swimming ability at increasing dose.

GRAPHICAL ABSTRACT



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ABSTRACT

Aluminium and cadmium are biologically non-essential metals with a role in neurodegenerative and neuromuscular diseases. As an attractive model for neurobehavioural studies, zebrafish at 6 h post fertilization were exposed to 9, 18, 36 and 72 μM $CdCl_2$ and 50, 100 and 200 μM $AlCl_3$, respectively, for 72 h, and motility such as distance moved, mean velocity, cumulative movement, meander and heading were measured by DanioVision equipment. The hatching time was also analysed. A delay in the exit from the chorion was observed in all treated larvae with respect to the controls. $CdCl_2$ acted on the exit from the chorion of larvae with a dose-dependent delay. By contrast, the delay caused by $AlCl_3$ was greater at low concentrations. A dose-dependent reduction in swimming performance was observed in the larvae exposed to $CdCl_2$. Instead, for those exposed to $AlCl_3$, swimming performance improved at higher concentrations although values were in general lower than those of control. All the parameters had a similar trend except the meander parameter which showed a dose-dependent reduction. These data show that cadmium and aluminium can delay hatching and alter swimming ability in the early developmental stages of zebrafish, albeit with different effects, suggesting that exposure to sublethal concentrations of both metals can change behavioural parameters.

1. Introduction

Aluminium (Al) and cadmium (Cd) are toxic metals, ubiquitous in the environment and subject to bioaccumulation (Favorito et al.,

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2011; Guo et al., 2007; Pereira et al., 2013; Suruchi and Pankaj, 2011). Cadmium is a highly toxic non-essential heavy metal and its adverse influence on oxidative stress is well recognized (Irfan et al., 2013). Cd can be toxic even at low doses since it accumulates and has a long biological half-life. In recent years, Cd has also been indicated as a possible aetiological factor in neurodegenerative diseases (Chin-Chan et al., 2015). It affects the nervous system by producing reactive oxygen species, changes in transmitter release (Antonio et al., 1999) and blood–brain barrier alterations (Shukla et al., 1996), but it also induces changes in calcium homeostasis leading to DNA damage and lipid peroxidation (Kumar et al., 1996; Lopez et al., 2006). Aluminium is the third most abundant element found in the earth's crust (Gupta et al., 2013). It occurs naturally in the air, water and soil. Some investigations on environmental toxicology revealed that aluminium might present a high risk for humans, animals and plants, causing many diseases (Barabasz et al., 2002). At high concentrations Al is very toxic for aquatic animals, especially for gill-breathing organisms such as fish (Rosseland et al., 1990). Al and Cd have been recognized as very harmful for the nervous system (Monaco et al., 2016, 2017a), influencing neurodegenerative and neuromuscular diseases (Jomova et al., 2010). They appear to be among the contributors of amyotrophic lateral sclerosis (Bar-Sela et al., 2001; Flaten, 1990) and Parkinson's disease (Okuda et al., 1997; Yasui et al., 1992), whilst representing a risk factor for the development of Alzheimer's disease (AD) (Notarachille et al., 2014; Panayi et al., 2002; Tomljenovic, 2011). In particular, the greatest complications of aluminium are neurotoxicity effects such as neuronal atrophy in the locus coeruleus, substantia nigra and striatum (Jaishankar et al., 2014). Nevertheless, some critical points in the mechanisms of Al neurotoxic actions are still unclear. While the mechanism of cadmium is not clearly understood either, its ionic shape (Cd^{2+}) is known to interfere with the release of acetylcholine from motor neuron terminals (Jaishankar et al., 2014). In the present work the effects of aluminium and cadmium were investigated on zebrafish larvae since negative effects on the development of this fish have been shown elsewhere (Monaco et al., 2017a, 2017b; Yin et al., 2017; Zhu et al., 2008). In addition, the zebrafish is an experimental model that has recently become the focus of neurobehavioural studies (Senger et al., 2011).

The zebrafish *(Danio rerio)* like other teleosts (Ferrandino and Grimaldi, 2008; Gay et al., 2016) and on a par with different animals (Galdiero et al., 2017; Libralato et al., 2017) is an optimum animal model for toxicology studies of environmental exposure. It is an attractive model of neurobehavioural studies since the larvae show neuropathological behaviour that is quantifiable and relate to those seen in humans (Best and Alderton, 2008; Guo, 2004). Moreover, the zebrafish has optically transparent eggs, a rapid development with the major organs that are evident within 24 h post-fertilization (hpf) (D'Costa and Shepherd, 2009; Kimmel et al., 1995). In previous studies we showed some neurotoxic effects of cadmium and aluminium in adult zebrafish (Favorito et al., 2011; Monaco et al., 2016) and during their embryological development (Monaco et al., 2017a, 2017b) but also in lizards (Favorito et al., 2010, 2017; Ferrandino et al., 2009). The aim of this work was therefore to continue in this direction and to study the behaviour of zebrafish larvae after exposure to the above metals, analysing the speed of hatching from the chorion and standard parameters of swimming ability. Swimming ability is indicative of motility performance, in turn indicating the normal development of larvae. In our study, motility was revealed by DanioVision equipment (Noldus) considering the reported parameters and described in accordance with EthoVision XT 10 technical specifications: *Distance moved*, that is distance travelled by a body point from the previous sample to the current one; *Mean velocity*, the distance travelled by the body point per unit of time; *Cumulative movement*, which describes two possible states, 'moving' and 'not moving', indicating whether the spatial movement of a body is within speed thresholds; *Heading,* the parameter on the direction of movement of a body point between one sample and the previous, relative to a line parallel to the x axis in the coordinate system; *Meander,* which measures the change in direction of a body point, relative to the distance moved. These analyses were conducted on embryos at 6 hpf (hours post fertilization) and exposed for 72 h to sublethal concentrations of aluminium chloride ($AlCl_3$) (50, 100 and 200 μM) and cadmium chloride ($CdCl_2$) (9, 18, 36 and 72 μM).

2. Materials and methods

2.1. Embryo growth

Embryos were generated from *Danio rerio* adult fish housed in well-oxygenated tanks, with a natural photoperiod of 12 h:12 h light/dark, at a temperature of 28.5 °C and a pH of 7.6 (Westerfield, 2000). The embryos were collected and housed with E3 medium at 28.5 °C. At 6 hpf they were selected for experimental procedures. All the experiments were performed in accordance with the Guidelines for Animal Experimentation of the Italian Department of Health.

2.2. Chemicals

For cadmium treatment a stock solution of 25 mM $CdCl_2 \cdot H_2O$ (Carlo Erba, Cornaredo, MI, Italy, CAS no. 35658-65-2) was prepared and by scalar dilutions $CdCl_2$ at 9, 18, 36 and 72 μM was obtained. For aluminium treatment, from a stock solution of $AlCl_3 \cdot 6H_20$ 8 mM (Carlo Erba, Italy; CAS no. 7784-13-6), a series of solutions was prepared at concentrations of 50, 100 and 200 μM. The concentrations used are sub-lethal in light of the survival curves of embryos tested in previous studies for both metals (Meinelt et al., 2001; Monaco et al., 2017a).

2.3. Embryo treatment

Embryo (6 hpf) treatments were conducted in six-well plates at a density of 10 embryos per well incubated at 28.5 °C. Each well contained 10 mL of test solutions renewed per day. Embryos were exposed to 50, 100 and 200 μM of $AlCl_3$ and to 9, 18, 36 and 72 μM of $CdCl_2$. For each concentration 50 embryos were used; 50 other embryos were for controls simply exposed to the breeding solution. All treatments were carried out for 72 h and the larvae at 78 hpf (Kimmel et al., 1995) were analysed for the study of their dechorionation time and to evaluate motility ability.

2.4. Rate of dechorionation

Rate of larvae dechorionation was determined by counting the number of larvae exiting from the chorion with respect to those in the chorion at 72 h from the start of exposure (78 hpf) both with $CdCl_2$ and with $AlCl_3$ for each element concentration by means of a Leica Zoom 2000 stereomicroscope at a magnification of 10X. The average values were obtained from experiments performed in triplicate and statistically validated.

2.5. Motility assay

Motility assay was performed by DanioVision (Noldus, Wageningen, The Netherlands), which allows the motility of zebrafish larvae to be evaluated, through a system equipped with an observation chamber that monitors the motor activity of a maximum of

96 individuals at the same time. The tool includes both software, namely EthoVision XT, and equipment that can control the temperature inside the instrument and provide test results in a very short time. The data are obtained thanks to a computer connected to DanioVision to process the video tracking the movements, the graphs related to the variables analysed and the data for each variable. The measured variables were distance moved, mean velocity, cumulative movement, meander and heading. Twenty larvae of zebrafish, with regular phenotype, for each experimental group, both $AlCl_3$ and of $CdCl_2$ and a group of control, were analysed at 78 hpf. All the experiments were replicated three times. Each single larva was placed individually in 96 multi-well plates with 200 μL of breeding solution. The study was conducted at a constant temperature of 28.5 °C for a total of 20 min. During the entire analysis, individuals were struck by a light which, for the first 10 min, was required for their adaptation in the well and, for the remaining 10 min, for the actual analysis. The system was optimised in order to eliminate variations due to different well location effects when comparing groups of animals subject to various treatments. *Distance moved* indicates the average distance travelled by the larvae for each treatment with respect to the average distance travelled by the control larvae. It was measured in millimetre paths (mm).

Mean velocity indicates the average speed measured in mm/s of the larvae for each treatment compared with the average speed of the control larvae.

Cumulative movement indicates the total time of the movements and was measured in seconds (s).

Heading identifies the larvae's movement from the x axis, and it is measured like the average number of collisions of samples against the wall.

Meander is the parameter that measures the change in direction of a body point relative to the distance moved.

2.6. Statistical analysis

Data were processed by means of GraphPad-Prism 7. Rate of dechorionation and the differences in swimming ability between control and treated larvae were tested by ANOVA test ($P = 0.05$) followed by Tukey's post hoc test. When studying the behavioural effects of $CdCl_2$ and $AlCl_3$ on zebrafish by means of a high-throughput tracking system, statistical analyses were generated by the DanioVision (Noldus) software system. Data are mean ± SD: $P < 0.05$ was considered to be statistically significant (*), $P < 0.01$ very significant (**) and $P < 0.001$ highly significant (***).

3. Results

3.1. Rate of dechorionation

The numbers of larvae able to exit the chorion were analysed at 72 h from treatment both with $CdCl_2$ and with $AlCl_3$ for each concentration. Before 72 h all treated larvae were still in the chorion. Instead, by the above development stage all the control larvae had come out of the chorion as occurs physiologically. For the treatment of cadmium the rate of larvae outside the chorion decreased significantly with the increase in the concentration of Cd solutions: the proportion of larvae hatching was 83% ± 0.07 at 9 μM, 76% ± 0.05 at 18 μM, 54% ± 0.05 at 36 μM and only 35% ± 0.07 at 72 μM. Instead for Al, the dechorionation rate increased together with Al concentrations, albeit always remaining lower than that of the controls: dechorionated larvae amounted to 57% ± 0.06 at 50 μM, 70% ± 0.06 at 100 and 83% ± 0.04 at 200 μM of $AlCl_3$, respectively (Fig. 1).

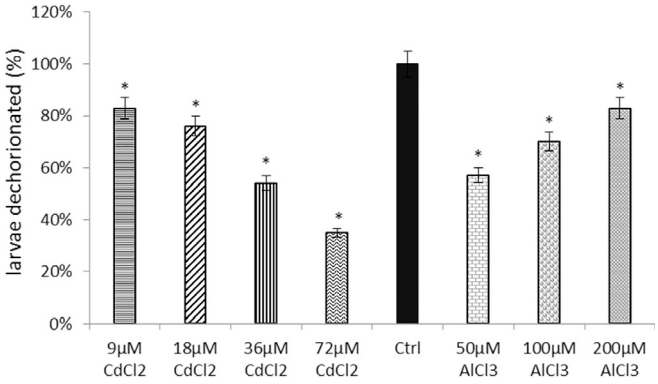

Fig. 1. Graphical representation of the dechorionation rate of control and larvae treated for 72 h with $CdCl_2$ (9 μM, 18 μM, 36 μM and 72 μM) and with $AlCl_3$ (50 μM, 100 μM and 200 μM). *P < 0.05.

3.2. Motility assay

The average distance travelled by the control larvae (340.7 ± 17.10 mm) was greater than that of larvae treated both with $CdCl_2$ and with $AlCl_3$. Treatment with $CdCl_2$ influenced the distance moved; in fact the travelled millimetres showed a general decrease dose-dependent. Indeed, upon increasing the concentration of cadmium this parameter showed a proportional decrease: 106.0 ± 5.30 mm at 9 μM, 79.5 ± 3.97 mm at 18 μM, 71.0 ± 3.55 mm at 36 μM and at 72 μM only 30.6 ± 1.53 mm. Exposure to $AlCl_3$ induced the opposite variations to those of cadmium: at 50 μM $AlCl_3$ the distance travelled was 22.5 ± 1.11 mm, 51.6 ± 2.58 mm at 100 μM and 200.34 ± 10.1 mm at 200 μM. The values were lower than those of the control larvae but instead with an inversion of performance: upon increasing the concentration of aluminium the larvae showed an increase in distance travelled. In the graph (Fig. 2) the average distances are summarised.

The average speed of the control larvae (0.79 ± 0.29 mm/s) exceeded that of those treated. It was significantly lower for those exposed to cadmium: at 9 μM $CdCl_2$ the mean velocity was 0.24 ± 0.08 mm/s but lower at other concentrations, for which the values were relatively constant (0.08 ± 0.03 mm/s at 18 μM, 0.09 ± 0.04 mm/s at 36 μM and 0.08 ± 0.03 mm/s at 72 μM). Larvae treated with $AlCl_3$ showed a lower mean velocity than the control, although the values in question increased with the aluminium concentration: at 50 μM mean velocity was 0.10 ± 0.03 mm/s, at 100 μM it was 0.43 ± 0.15 mm/s and at 200 μM it was 0.68 ± 0.14 mm/s (Fig. 3).

This value was lower for larvae treated with cadmium: at 9 μM $CdCl_2$ mean cumulative movement was 16.81 ± 0.84 s compared

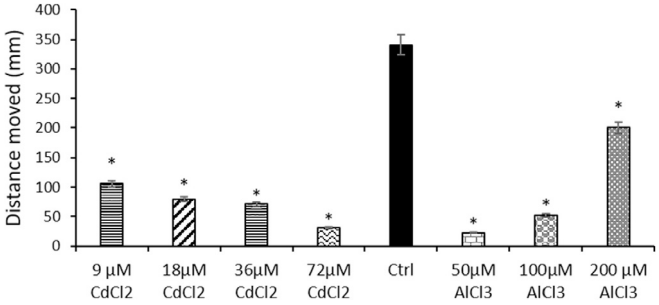

Fig. 2. Graphical representation of the average *distance moved* (mm) of the control larvae and larvae treated for 72 h, respectively with $CdCl_2$ (9 μM, 18 μM, 36 μM and 72 μM) and with $AlCl_3$ (50 μM, 100 μM and 200 μM). P values < 0.05(*).

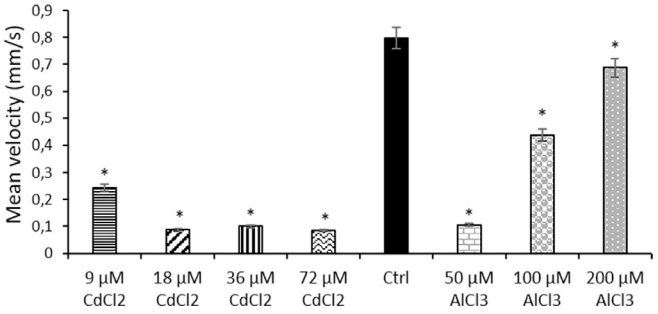

Fig. 3. Graphical representation of the *mean velocity* (mm/s) of the control larvae and larvae treated for 72 h, respectively, with $CdCl_2$ (9 µM, 18 µM, 36 µM and 72 µM) and with $AlCl_3$ (50 µM, 100 µM and 200 µM). P values < 0.05(*).

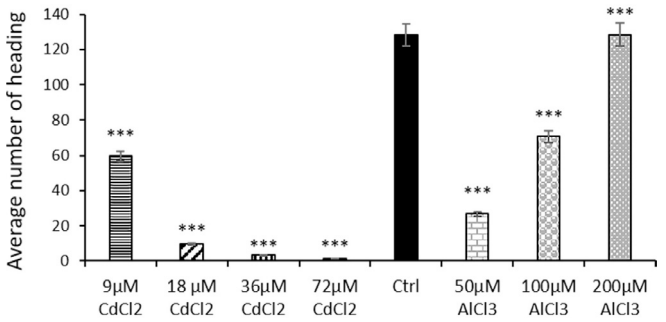

Fig. 5. Graphical representation of the average *heading* of control larvae and those treated for 72 h with $CdCl_2$ (9 µM, 18 µM, 36 µM and 72 µM) and with $AlCl_3$ (50 µM, 100 µM and 200 µM). P values < 0.001(***).

with the mean of control larvae (76.52 ± 3.82 s) and decreased further for those treated at 18 µM of $CdCl_2$ (8.77 ± 0.43 s) and at successive concentrations, for which was also relatively constant values were observed (9.86 ± 0.49 s at 36 µM and 9.37 ± 0.46 s at 72 µM, respectively). For the larvae treated with $AlCl_3$ the total time of movements exceeded those treated with Cd, although it was less than that of the control (19 ± 0.95 s at 50 µM, 23.36 ± 1.16 s at 100 µM and 32.24 ± 1.61 s at 200 µM, as shown in Fig. 4).

A large number of headings are a symptom of the good state of the larvae, as it indicates that they had greater motility and that they repeatedly reached the wall of the well during the observation time. Compared with the controls the number of collisions decreases significantly with the increase in Cd concentration in solution. This value is in agreement with the velocity parameters and distance moved. Indeed, the control larvae with an average of 128.28 ± 6.41 showed twice the number of headings as those treated at 9 µM of $CdCl_2$ (59.56 ± 2.97). At 18 µM of $CdCl_2$ this parameter was 9.76 ± 0.48, at 36 µM it was 3.19 ± 0.15 and at 72 µM it was 1.01 ± 0.05. Larvae exposed to aluminium showed a greater number of collisions than those treated with Cd, which increased with the metal concentration: at 50 µM of $AlCl_3$ the headings were 26.72 ± 1.33 and at 100 µM they numbered 70.60 ± 3.53. At 200 µM of $AlCl_3$ larvae collisions against the wall amounted to 128.47 ± 6.42, a value similar to the numbers of collisions in control larvae (Fig. 5).

For this measure the positive values indicate the continuous and uniform movements in only one direction from the centre to the ends of the well for a few milliseconds. By contrast, the negative values represent the rapid changes, neither continuous nor uniform, that occur in the same milliseconds. Uniform movements are typical of larvae in good health, whereas rapid changes in direction are symptoms of a compromised locomotion system. The data showed a smaller average number of unchecked movements of the controls with respect to all the treated larvae. The trend of the data for Cd appeared to overlap other motility parameters: at 9 µM, 18 µM, 36 µM and 72 µM of $CdCl_2$ the values of uniform movements were −1.79 ± 0.08, −12.29 ± 0.61, −21.3 ± 1.06, and −48.85 ± 2.44, respectively, instead the mean of control larvae was 93.45 ± 4.67. A different response was noted for the Al treatment: no overlapping of the trend was revealed with the parameters already described. In the latter case the number of unchecked movements increased at higher concentrations: uniform movements amounted to 64.23 ± 3.21 at 50 µM, −17.42 ± 0.87 at 100 µM and −43.92 ± 2.19 at 200 µM, as shown in Fig. 6.

4. Discussion

In our previous reports we showed that exposure to cadmium and aluminium induced alterations on embryonic development, changes in heart rate assessment (Monaco et al., 2017a) and neurodegenerative effects (Favorito et al., 2011; Monaco et al., 2016, 2017b). In the light of this we analysed some behavioural parameters like hatching time and swimming ability in developing zebrafish after exposure to sublethal concentrations tested in previous studies for both metals (Meinelt et al., 2001; Monaco et al., 2017a). In this study, the dechorionation rates showed a general delay for all treated larvae with respect to the controls. If at 78 hpf all control larvae were exited from chorion, this did not happen for those exposed to cadmium or to aluminium. Indeed, the number of larvae exiting the chorion decreased significantly with the increase in $CdCl_2$ concentration. Also for the Al treatment, the values were always lower than those found in control larvae, but the number of eggs hatching was higher at high concentrations of $AlCl_3$ (100 and 200 µM) than that observed at a low concentration (50 µM). The

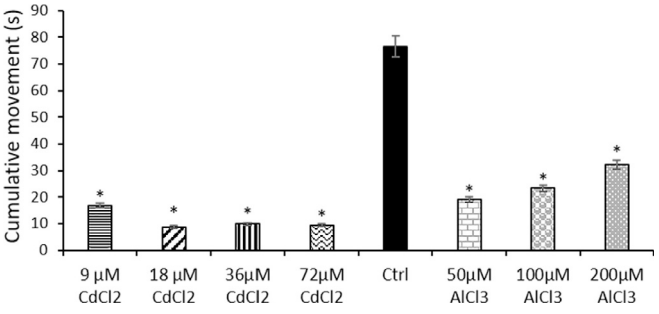

Fig. 4. Graphical representation of average *cumulative movement* of the control larvae and those treated for 72 h with $CdCl_2$ (9 µM, 18 µM, 36 µM and 72 µM) and with $AlCl_3$ (50 µM, 100 µM and 200 µM). P values < 0.05(*).

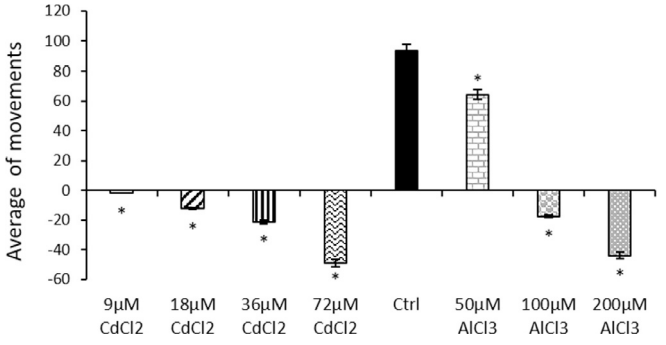

Fig. 6. The graph represents the *meander* values of control larvae and those treated with $CdCl_2$ and $AlCl_3$ after 72 h of exposure with $CdCl_2$ (9 µM, 18 µM, 36 µM and 72 µM) and with $AlCl_3$ (50 µM, 100 µM and 200 µM). P values < 0.05(*).

hatching of larvae is an important point in the life cycle of fish, and a combination of biochemical and physical mechanisms regulates the process, which happens physiologically between 48 hpf and 72 hpf (Kimmel et al., 1995; Wang et al., 2018). This process is achieved by the secretion of choriolysin but also by spontaneous movements of the developing embryos (De la Paz et al., 2017; Schoots et al., 1982). We hypothesised that the frequency of spontaneous movement of embryos within the chorion was the reason for the general delay in the hatching process, rather than other factors that would alter the production of choriolysin, because the hatching time overlapped with the trend of other motility parameters measured for both metals. For the effects induced by Cd, the regular and repeated dose-dependent pattern of all the parameters analysed (distance moved, mean velocity, cumulative movement, heading and meander) suggests a common mechanism underlying such alterations.

Instead, the response to aluminium was conflicting: the delay of hatching shown may be superimposed upon all motility values except the meander parameter for which the trend was completely reversed. Moreover, treatment with $AlCl_3$ also induced partial recovery of physiological activity as the metal concentration increased while remaining below the performance of controls.

To our knowledge, this is the first time the abnormal behaviour induced by Cd and Al in larval zebrafish has been compared and very little is known about the mechanisms activated. For the limited motility of larvae exposed to cadmium, the data were consistent with similar experiments in which Cd is reported to have induced decreases in swimming ability upon increasing the dose (Tu et al., 2017; Zhang et al., 2016). Such effects were probably caused by alterations in organisation, composition and function of the skeletal muscle fibre with a significant reduction in swimming performance (Avallone et al., 2015a). Furthermore, exposure to Cd makes the larvae so lethargic that they get tired quickly. This is a probable consequence of glycogen reserves being compromised (Pierron et al., 2007) even if such effects could be due to the direct interference of Cd on glycolytic enzymes, lactate dehydrogenase, hexokinase and phosphofructokinase (Almeida et al., 2001; Ramirez-Bajo et al., 2014). While much has been written about cadmium, little is known about aluminium toxicity. However, Al is known to cause a reduction in the neuronal stem cell pool, thereby limiting the neuroblast differentiation process (Nam et al., 2016), as well as an alteration of the glucose metabolism (Wei et al., 2018). The above factors could be the cause of the impairment of swimming ability that we observed. The stronger performance of larvae exposed to aluminium is corroborated by work on the Atlantic salmon exposed to acidic water and Al in which it was demonstrated that the treated fish were more active than the control exposed to circumneutral water (Brodeur et al., 2001). Conversely, in Al-exposed juvenile rainbow trout reduced swimming activity was observed (Allin and Wilson, 1999). That said, these contrasting findings are indicative that fish of different species can react differently to sublethal levels of aluminium. The atypical response to exposure to high concentrations of aluminium, for which the larvae appeared more reactive than those exposed to low concentrations of the metal, was revealed in all parameters except meander. This is in agreement with a study on zebrafish larvae which increased their movement speed at high Al concentrations (Chen et al., 2017), and another on mice, which at low Al concentrations decreased locomotor activity, but at high concentrations showed neither neurobehavioural changes with respect to controls (Crépeaux et al., 2017). Selective toxicity of the lowest dose may well challenge the classic toxicology adage “the dose makes the poison” because not response to classic mechanism dose-dependent. In fact only at high concentrations are actived adaptive immune responses (Seubert et al., 2008) with unknown threshold-dose mechanism again. It could be that only low concentrations cause the activation of mechanisms of alterations which are then turned off with higher concentrations, as reported in adult zebrafish treated with a high dose of cadmium (Avallone et al., 2015b). The future hypothesis, to be demonstrated, is that low levels of metal do not fully activate protective mechanisms, thereby allowing extensive but not lethal damage.

For the *Meander* parameter, the trend of values is different because both with increasing concentrations of Cd and with increasing Al concentrations, the values get worse, that is the number of unchecked movements increase with the concentrations. This parameter for Al is most important because unchecked movements are typical of neurologic pathologies like Parkinson's disease, suggesting a possible correlation between the activated mechanisms (Saberzadeh et al., 2016). Although its neurotoxic effects are still not completely understood, the literature suggests that aluminium interacts with the cholinergic system, acting as a cholinotoxin (Gulya et al., 1990) and increasing Acetylcholinesterase activity in *Danio rerio* (Senger et al., 2011). Cholinergic systems are known to be involved in locomotor activity responses to novel stimuli and in the performance of spatial memory tasks (Pepeu and Giovannini, 2004). Moreover, to support this theory, new drugs, such as cholinesterase inhibitors, have been used in patients to block Parkinson's disease (Rolinski et al., 2012). Our results suggest the importance of continued in-depth study into such alterations of motility for their environment implications, given the presence of Cd and Al in polluted waters (Wilbers et al., 2014; Zhao et al., 2018), since alterations in fish behaviour serve as a sensitive tool for analysing toxicological impact and environmental risk (Si et al., 2017). Reduced motility can limit fish survival in the wild (Brodeur et al., 2001), where reduced swimming activity can affect the ability of fish to avoid predation and successfully reproduce (Allin and Wilson, 1999).

Moreover, further experiments are also required to shed light on the mechanisms underlying delay of motility, due to possible human implications of such research. In this context, using zebrafish as a model system would appear advisable, given that this fish now provides an excellent complement to the mouse to study the factors of development and muscular and neurological functions (Bretaud et al., 2007; Guo, 2004; Matsui and Takahashi, 2018). In addition, the correlation between neurodegenerative diseases like Parkinson's and Alzheimer's and exposure to Cd and Al needs to be elucidated, given that exposure has been suggested as a risk factor in the onset of such diseases (Jomova et al., 2010), especially since the mechanism behind the correlation has yet to be fully clarified.

5. Conclusion

The present work showed that aluminium and cadmium have serious effects on the behaviour of developing zebrafish. We demonstrated that exposure of embryos at 6 hpf to 9 μM, 18 μM, 36 μM and 72 μM $CdCl_2$ resulted in a delay in larval hatching time which increased with elevated concentrations. Cd also induced a significant reduction in the movement of individuals at the larval stage (78 hpf), always with the same trend and affecting the parameters of behaviour analysed by DanioVision. This reduction in swimming performance may be related to alterations of skeletal muscle fibre but also of compromised glycogen reserves. In addition, the results on embryos exposed to increasing sub-lethal $AlCl_3$ concentrations (50 μM, 100 μM, 200 μM) showed a decrease in swimming performance when the embryos were exposed to low concentrations and a recovery in such functions in those exposed to high concentrations, which do not however reach the values observed in the control organisms. This effect occurred both for

hatching time, and for all parameters studied but not for the meander parameter. In this case the performance of larvae worsens with increased concentrations of $AlCl_3$. The behaviour of zebrafish larvae exposed to aluminium appears atypical with respect to that observed with cadmium. These results indicate the toxic effects of Cd and Al at sublethal concentrations on embryonic development of *D. rerio* and suggest the need for further experiments to elucidate the different mechanisms underlying such alterations.

Declarations of interest

The authors have no conflict of interest to declare.

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References

Allin, C., Wilson, R., 1999. Behavioural and metabolic effects of chronic exposure to sublethal aluminum in acidic soft water in juvenile rainbow trout (Oncorhynchus mykiss). Can. J. Fish. Aquat. Sci. 56 (4), 670–678. https://doi.org/10.1139/f99-002.

Almeida, J.A., Novelli, E.L., Dal Pai Silva, M., Alves, R.J., 2001. Environmental cadmium exposure and metabolic responses of the Nile tilapia Oreochromis niloticus. Environ. Pollut. 114, 169–175. https://doi.org/10.1016/S0269-7491(00)00221-9.

Antonio, M.T., Corpas, I., Leret, M.L., 1999. Neurochemical changes in newborn rat's brain after gestational cadmium and lead exposure. Toxicol. Lett. 104, 1–9.

Avallone, B., Agnisola, C., Cerciello, R., Panzuto, R., Simoniello, P., Cretì, P., Motta, C.M., 2015a. Structural and functional changes in the zebrafish *(Danio rerio)* skeletal muscle after cadmium exposure. Cell Biol. Toxicol. 31 (6), 273–283. https://doi.org/10.1007/s10565-015-9310-0.

Avallone, B., Crispino, R., Cerciello, R., Simoniello, P., Panzuto, R., Motta, C.M., 2015b. Cadmium effects on the retina of adult *Danio rerio*. C. R. Biol. 338 (1), 40–47. https://doi.org/10.1016/j.crvi.2014.10.005.

Bar-Sela, S., Reingold, S., Richter, E.D., 2001. Amyotrophic lateral sclerosis in a battery-factory worker exposed to cadmium. Int. J. Occup. Environ. Health 7 (2), 109–112. https://doi.org/10.1179/107735201800339470.

Barabasz, W., Albinska, D., Jaskowska, M., Lipiec, J., 2002. Ecotoxicology of aluminium. Pol. J. Environ. Stud. 11 (3), 199–203.

Best, J.D., Alderton, W.K., 2008. Zebrafish: an in vivo model for the study of neurological diseases. Neuropsychiatr. Dis. Treat. 4 (3), 567–576.

Bretaud, S., Allen, C., Ingham, P.W., Bandmann, O., 2007. p53-dependent neuronal cell death in a DJ-1-deficient zebrafish model of Parkinson's disease. J. Neurochem. 100 (6), 1626–1635. https://doi.org/10.1111/j.1471-4159.2006.04291.x.

Brodeur, J., Økland, F., Finstad, B., Dixon, D.G., Scott McKinley, R.S., 2001. Effects of subchronic exposure to aluminium in acidic water on bioenergetics of Atlantic salmon (*Salmo salar*). Ecotoxicol. Environ. Saf. 49, 226–234. https://doi.org/10.1006/eesa.2001.2054.

Chen, J.P., Shang, N., He, K.H., Zhang, L., Niu, Q., Zhang, Q.L., 2017. Influence of aluminum chloride exposure on embryonic development of zebrafish and neurobehavior of juvenile fish. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi 35 (3), 166–170. https://doi.org/10.3760/cma.j.issn.1001-9391.2017.03.002, 20.

Chin-Chan, M., Navarro-Yepes, J., Quintanilla-Vega, B., 2015. Environmental pollutants as risk factors for neurodegenerative disorders: Alzheimer and Parkinson diseases. Front. Cell. Neurosci. 9, 124. https://doi.org/10.3389/fncel.2015.00124.

Crépeaux, G., Eidi, H., David, M.O., Baba-Amer, Y., Tzavara, E., Giros, B., Authier, F.J., Exley, C., Shaw, C.A., Cadusseau, J., Gherardi, R.K., 2017. Non-linear dose-response of aluminium hydroxide adjuvant particles: selective low dose neurotoxicity. Toxicology 15 (375), 48–57. https://doi.org/10.1016/j.tox.2016.11.018.

D'Costa, A., Shepherd, I.T., 2009. Zebrafish development and genetics: introducing undergraduates to developmental biology and genetics in a large introductory laboratory class. Zebrafish 6 (2), 169–177. https://doi.org/10.1089/zeb.2008.0562.

De la Paz, J.F., Beiza, N., Paredes-Zúñiga, S., Hoare, M.S., Allende, M.L., 2017. Triazole fungicides inhibit zebrafish hatching by blocking the secretory function of hatching gland cells. Int. J. Mol. Sci. 18, 710. https://doi.org/10.3390/ijms18040710.

Favorito, R., Grimaldi, M.C., Coppola, M., Ferrandino, I., 2010. Effects of acute cadmium exposure on the pituitary gland of *Podarcis sicula*. Open Zool. J. 3, 30–36. https://doi.org/10.2174/1874336601003010030.

Favorito, R., Chiarelli, G., Grimaldi, M.C., De Bonis, S., Lancieri, M., Ferrandino, I., 2011. Bioaccumulation of cadmium and its cytotoxic effect on zebrafish brain. Chem. Ecol. 27 (2), 39–46. https://doi.org/10.1080/02757540.2011.625937.

Favorito, R., Monaco, A., Grimaldi, M.C., Ferrandino, I., 2017. Effects of cadmium on the glial architecture in lizard brain. Eur. J. Histochem. 61, 2734. https://doi.org/10.4081/ejh.2017.2734.

Ferrandino, I., Grimaldi, M.C., 2008. Ultra-structural study of the pituicytes in the pituitary gland of the teleost Diplodus sargus. Brain Res. Bull. 75 (1), 133–137. https://doi.org/10.1016/j.brainresbull.2007.08.006.

Ferrandino, I., Favorito, R., Annunziata, M., Grimaldi, M.C., 2009. Cadmium induces apoptosis in the pituitary gland of *Podarcis sicula*. Ann. N. Y. Acad. Sci. 1163, 386–388. https://doi.org/10.1111/j.1749-6632.2008.03667.x.

Flaten, T.P., 1990. Geographical associations between aluminium in drinking water and death rates with dementia (including Alzheimer's disease), Parkinson's disease and amyotrophic lateral sclerosis in Norway. Environ. Geochem. Health 12 (1–2), 152–167. https://doi.org/10.1007/BF01734064.

Galdiero, E., Falanga, A., Siciliano, A., Maselli, V., Guida, M., Carotenuto, R., Tussellino, M., Lombardi, L., Benvenuto, G., Galdiero, S., 2017. *Daphnia magna* and *Xenopus laevis* as in vivo models to probe toxicity and uptake of quantum dots functionalized with gH625. Int. J. Nanomed. 4 (12), 2717–2731. https://doi.org/10.2147/IJN.S127226.

Gay, F., Ferrandino, I., Monaco, A., Cerulo, M., Capasso, G., Capaldo, A., 2016. Histological and hormonal changes in the European eel (Anguilla anguilla) after exposure to environmental cocaine concentration. J. Fish. Dis. 39 (3), 295–308. https://doi.org/10.1111/jfd.12362.

Gulya, K., Rakonczay, Z., Kasa, P., 1990. Cholinotoxic effects of aluminum in rat brain. J. Neurochem. 54, 1020–1026. https://doi.org/10.1111/j.1471-4159.1990.tb02352.x.

Guo, S., 2004. Linking genes to brain, behavior and neurological diseases: what can we learn from zebrafish? Genes Brain Behav. 3 (2), 63–74. https://doi.org/10.1046/j.1601-183X.2003.00053.x.

Guo, T.R., Zhang, G.P., Zhang, Y.H., 2007. Physiological changes in barley plants under combined toxicity of aluminum, copper and cadmium. Colloids Surf. B. Biointerfaces. 57 (2), 182–188. https://doi.org/10.1016/j.colsurfb.2007.01.013.

Gupta, P.N., Pillai, S.B., Ahmad, S.Z., Babu, S.M., 2013. New onset S wave in pulmonary embolism: revisited (something old and something new). BMJ Case Rep. https://doi.org/10.1136/bcr-2013-200733 pii: bcr2013200733.

Irfan, M., Hayat, S., Ahmad, A., Alyemeni, M.N., 2013. Soil cadmium enrichment: allocation and plant physiological manifestations. Saudi J. Biol. Sci. 20 (1), 1–10. https://doi.org/10.1016/j.sjbs.2012.11.004.

Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B.B., Beeregowda, K.N., 2014. Toxicity, mechanism and health effects of some heavy metals. Interdiscip. Toxicol. 7 (2), 60–72. https://doi.org/10.2478/intox-2014-0009.

Jomova, K., Vondrakova, D., Lawson, M., Valko, M., 2010. Metals, oxidative stress and neurodegenerative disorders. Mol. Cell. Biochem. 345 (1–2), 91–104. https://doi.org/10.1007/s11010-010-0563-x.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310.

Kumar, R., Agarwal, A.K., Seth, P.K., 1996. Oxidative stress-mediated neurotoxicity of cadmium. Toxicol. Lett. 89, 65–69. https://doi.org/10.1016/S0378-4274(96)03780-0.

Libralato, G., Galdiero, E., Falanga, A., Carotenuto, R., de Alteriis, E., Guida, M., 2017. Toxicity effects of functionalized quantum dots, gold and polystyrene nanoparticles on target aquatic biological models: a review. Molecules 22 (9), E1439. https://doi.org/10.3390/molecules22091439.

Lopez, E., Arce, C., Oset-Gasque, M.J., Canadas, S., Gonzalez, M.P., 2006. Cadmium induces reactive oxygen species generation and lipid peroxidation in cortical neurons in culture. Free Radic. Biol. Med. 40, 940–951. https://doi.org/10.1016/j.freeradbiomed.2005.10.062.

Matsui, H., Takahashi, R., 2018. Parkinson's disease pathogenesis from the viewpoint of small fish models. J. Neural. Transm. 125 (1), 25–33. https://doi.org/10.1007/s00702-017-1772-1.

Meinelt, T., Playle, R., Pietrock, M., Burnison, B.K., Wienke, A., Steinberg, C.E., 2001. Interaction of cadmium toxicity in embryos and larvae of zebrafish (*Danio rerio*) with calcium and humic substances. Aquat. Toxicol. 54 (3–4), 205–215. https://doi.org/10.1016/S0166-445X(01)00145-X.

Monaco, A., Grimaldi, M.C., Ferrandino, I., 2016. Neuroglial alterations in the zebrafish brain exposed to cadmium chloride. J. Appl. Toxicol. 36 (12), 1629–1638. https://doi.org/10.1002/jat.3328.

Monaco, A., Grimaldi, M.C., Ferrandino, I., 2017a. Aluminium chloride-induced toxicity in zebrafish larvae. J. Fish. Dis. 40 (5), 629–635. https://doi.org/10.1111/jfd.12544.

Monaco, A., Capriello, T., Grimaldi, M.C., Schiano, V., Ferrandino, I., 2017b. Neurodegeneration in zebrafish embryos and adults after cadmium exposure. Eur. J. Histochem. 61 (4), 28–33. https://doi.org/10.4081/ejh.2017.2833.

Nam, S.M., Kim, J.W., Yoo, D.Y., Kim, W., Jung, H.Y., Choi, J.H., Hwang, I.K., Seong, J.K., Yoon, Y.S., 2016. Effects of aluminum on the reduction of neural stem cells, proliferating cells, and differentiating neuroblasts in the dentate gyrus of D-galactose-treated mice via increasing oxidative stress. J. Vet. Sci. 17 (2), 127–136. https://doi.org/10.4142/jvs.2016.17.2.127.

Notarachille, G., Arnesano, F., Calò, V., Meleleo, D., 2014. Heavy metals toxicity: effect of cadmium ions on amyloid beta protein 1-42. Possible implications for Alzheimer's disease. Biometals 27 (2), 371–388. https://doi.org/10.1007/s10534-014-9719-6.

Okuda, B., Iwamoto, Y., Tachibana, H., Sugita, M., 1997. Parkinsonism after acute cadmium poisoning. Clin. Neurol. Neurosurg. 99 (4), 263–265. https://doi.org/

10.1016/S0303-8467(97)00090-5.
Panayi, A.E., Spyrou, N.M., Iversen, B.S., White, M.A., Part, P., 2002. Determination of cadmium and zinc in Alzheimer's brain tissue using inductively coupled plasma mass spectrometry. J. Neurol. Sci. 195 (1), 1–10. https://doi.org/10.1016/S0022-510X(01)00672-4.
Pepeu, G., Giovannini, M., 2004. Changes in acetylcholine extracellular levels during cognitive processes. Learn. Mem. 11 (1), 21–27. https://doi.org/10.1101/lm.68104.
Pereira, S., Cavalie, I., Camilleri, V., Gilbin, R., Adam-Guillermin, C., 2013. Comparative genotoxicity of aluminium and cadmium in embryonic zebrafish cells. Mutat. Res. 750 (1–2), 19–26. https://doi.org/10.1016/j.mrgentox.2012.07.007.
Pierron, F., Baudrimont, M., Bossy, A., Bourdineaud, J.P., Brèthes, D., Elie, P., Massabuau, J.,C., 2007. Impairment of lipid storage by cadmium in the European eel (*Anguilla anguilla*). Aquat. Toxicol. 81 (3), 3304–3311. https://doi.org/10.1016/j.aquatox.2006.12.014.
Ramırez-Bajo, M.J., De Atauri, P., Ortega, F.O., Westerhoff, H.V., Gelpí, J.L., Centelles, J.J., Cascante, M., 2014. Effects of cadmium and mercury on the upper part of skeletal muscle glycolysis in mice. PLoS One 9 (1), e80018. https://doi.org/10.1371/journal.pone.0080018.
Rolinski, M., Fox, C., Maidment, I., McShane, R., 2012. Cholinesterase inhibitors for dementia with Lewy bodies, Parkinson's disease dementia and cognitive impairment in Parkinson's disease. Cochrane Database Syst. Rev. 14 (3), CD006504. https://doi.org/10.1002/14651858.CD006504.pub2.
Rosseland, B.O., Eldhuset, T.D., Staurnes, M., 1990. Environmental effects of aluminium. Environ. Geochem. Health 12 (1–2), 17–27. https://doi.org/10.1007/BF01734045.
Saberzadeh, J., Arabsolghar, R., Takhshid, M.A., 2016. Alpha synuclein protein is involved in Aluminum-induced cell death and oxidative stress in PC12 cells. Brain Res. 1635, 153–160. https://doi.org/10.1016/j.brainres.2016.01.037.
Schoots, A.F.M., Stikkelbroeck, J.J.M., Bekhuis, J.F., Denucé, J.M., 1982. Hatching in teleostean fishes: fine structural changes in the egg envelope during enzymatic breakdown in vivo and in vitro. J. Ultrastruct. Res. 80 (2), 185–196. https://doi.org/10.1016/S0022-5320(82)90017-X.
Senger, M.R., Seibt, K.J., Cordenonzi, G., Dutra Dias, R., Bogo, M.R., Bonan, C.D., 2011. Aluminum exposure alters behavioral parameters and increases acetylcholinesterase activity in zebrafish *(Danio rerio)* brain. Cell Biol. Toxicol. 27 (3), 199–205. https://doi.org/10.1007/s10565-011-9181-y.
Seubert, A., Monaci, E., Pizza, M., O'Hagan, D.T., Wack, A., 2008. The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. J. Immunol. 180 (8), 5402–5412. https://doi.org/10.4049/jimmunol.180.8.5402.
Shukla, A., Shukla, G.S., Srimal, R.C., 1996. Cadmium-induced alterations in blood–brain barrier permeability and its possible correlation with decreased microvessel antioxidant potential in rat. Hum. Exp. Toxicol. 15, 400–405.
Si, J., Zhou, R., Song, J., Gan, L., Zhou, X., Di, C., Liu, Y., Mao, A., Zhao, Q., Wang, Y., Zhang, H., 2017. Toxic effects of 56Fe ion radiation on the zebrafish *(Danio rerio)* embryonic development. Aquat. Toxicol. 186, 87–95. https://doi.org/10.1016/j.aquatox.2017.02.028.
Suruchi, Pankaj, K., 2011. Assessment of heavy metal contamination in different vegetables grown in and around urban areas. RJET 5 (3), 162–179. https://doi.org/10.3923/rjet.2011.162.179.
Tomljenovic, L., 2011. Aluminum and Alzheimer's disease: after a century of controversy, is there a plausible link? J. Alzheimers Dis. 23 (4), 567–598. https://doi.org/10.3233/JAD-2010-101494.
Tu, H., Fan, C., Chen, X., Liu, J., Wang, B., Huang, Z., Zhang, Y., Meng, X., Zou, F., 2017. Effects of cadmium, manganese, and lead on locomotor activity and neurexin 2a expression in zebrafish. Environ. Toxicol. Chem. 36 (8), 2147–2154. https://doi.org/10.1002/etc.3748.
Wang, X.H., Souders, C.L., Zhao, Y.H., Martyniuk, C.J., 2018. Paraquat affects mitochondrial bioenergetics, dopamine system expression, and locomotor activity in zebrafish (*Danio rerio*). Chemosphere 191, 106–117. https://doi.org/10.1016/j.chemosphere.2017.10.032.
Wei, X., Wei, H., Yang, D., Li, D., Yang, X., He, M., Lin, E., Wu, B., 2018. Effect of aluminum exposure on glucose metabolism and its mechanism in rats. Biol. Trace Elem. Res. 1, 1–7. https://doi.org/10.1007/s12011-018-1318-x.
Westerfield, M., 2000. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*), fourth ed. University of Oregon Press, Eugene.
Wilbers, G.J., Becker, M., Nga, T., Sebesvari, Z., Renaud, F.G., 2014. Spatial and temporal variability of surface water pollution in the Mekong Delta, Vietnam. Sci. Total Environ. 485–486, 653–665. https://doi.org/10.1016/j.scitotenv.2014.03.049.
Yasui, M., Kihira, T., Ota, K., 1992. Calcium, magnesium and aluminum concentrations in Parkinson's disease. Neurotoxicology 13 (3), 593–600.
Yin, J., Wang, A.P., Li, W.F., Shi, R., Jin, H.T., Wei, J.F., 2017. Sensitive biomarkers identification for differentiating Cd and Pb induced toxicity on zebrafish embryos. Environ. Toxicol. Pharmacol. 56, 340–349. https://doi.org/10.1016/j.etap.2017.10.010.
Zhang, Y., Ma, J., Shi, L., Cao, D., Quan, X., 2016. Joint toxicity of cadmium and SDBS on *Daphnia magna* and *Danio rerio*. Ecotoxicology 25 (10), 1703–1711. https://doi.org/10.1007/s10646-016-1701-3.
Zhao, X.M., Yao, L., Ma, Q.L., Zhou, G.J., Wang, L., Fang, Q.L., Xu, Z.C., 2018. Distribution and ecological risk assessment of cadmium in water and sediment in Longjiang River, China: implication on water quality management after pollution accident. Chemosphere 194, 107–116. https://doi.org/10.1016/j.chemosphere.2017.11.127.
Zhu, X., Zhu, L., Duan, Z., Qi, R., Li, Y., Lang, Y., 2008. Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio)* early developmental stage. J. Environ. Sci. Health A Tox. Hazard Subst. Environ. Eng. 43 (3), 278–284. https://doi.org/10.1080/10934520701792779.

Paper in press (Aquatic Toxicology)

Apoptosis, oxidative stress and genotoxicity in developing zebrafish after aluminium exposure

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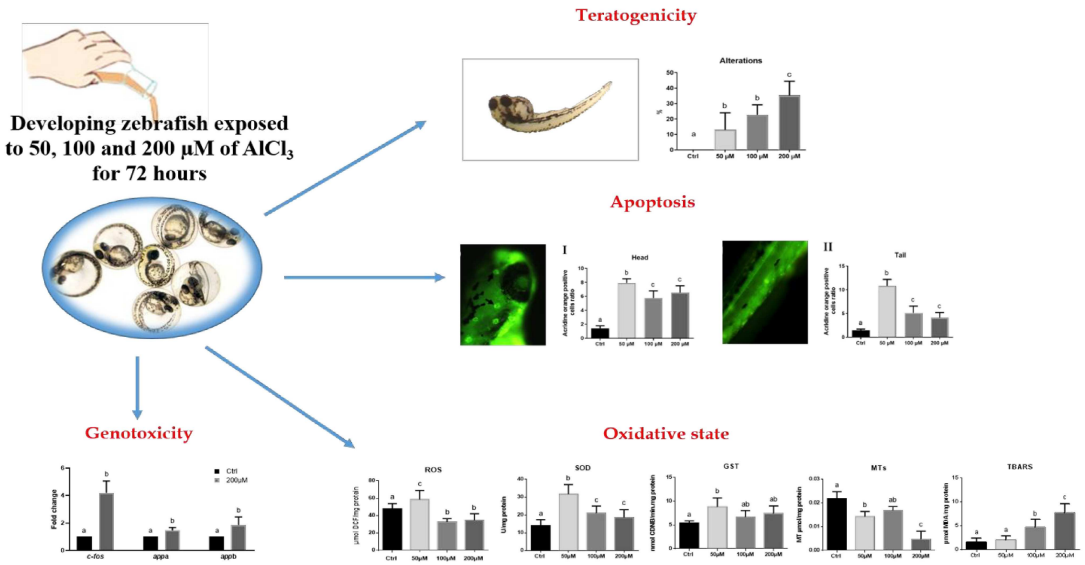
Graphical abstract



Abstract

Aluminium is a non-essential metal and potentially toxic to organisms whose environmental concentration increases due to pollution. In our previous studies, the behavioural changes induced by aluminium were already shown on zebrafish, a model organism widely used for ecotoxicology screening. To examine in depth the knowledge about the toxicity mechanism induced by this metal, zebrafish embryos, at 6 hpf, have been exposed to 50, 100 and 200 μM of $AlCl_3$ for 72 hours. Phenotypic alterations, apoptosis and oxidative stress responses have been assessed by evaluations of antioxidant defence and changes in metabolism at the end of treatment. The mRNA expression level of *c-fos*, *app*a and *app*b as marker genes of neural development and function were analysed by qPCR for the highest used concentration. The data showed that aluminium significantly affected the development of zebrafish inducing morphological alterations and cell death. The oxidative state of larvae was altered, although the formation of reactive oxygen species and the levels of metallothioneins, and the activity of some antioxidant enzymes, decreased at the maximum concentration tested. In addition, at this concentration, the expression of the evaluated genes increased. The comprehensive information obtained gives a realistic snapshot of the aluminium toxicity and provides new information on the mechanism of action of this metal.

Keywords: Antioxidants, gene expression, *Danio rerio* larvae, phenotypical alterations, oxidative biomarkers, Metallothioneins.

Introduction

Aluminium (Al) is a ubiquitous element naturally occurring in the soil, air, and water. Its presence in the environment has been increasing due to anthropogenic activities (Pereira et al., 2013) and the consequent pollution, especially in water bodies. It is well-known that the Al concentration in water bodies usually ranges from 0.001 to 0.05 mg/L when the pH is near-neutrality (WHO, 1997, 2013), but it may increase up to 50 mg/L in the polluted water due to acid rainfalls (ATSDR, 2008). The surface waters growing acidification induces the Al mobilisation from metastable compounds in

bottom sediments and its transition into water (Barabasz et al., 2002; Lawrence et al., 2007). As a result of its increase in the environment, the Al has been the subject of several studies in the last few recent years, demonstrating its toxicity for different organisms, both aquatic (Adams et al., 2018) and terrestrial (Coleman et al., 2010). It has been shown that Al can lead to adverse effects in different species, especially the aquatic organisms Indeed, it can alter the vertebrate organisms' survival and growth such as *D. rerio* (Monaco et al., 2017a) and *P. promelas* (Cardwell et al., 2018), and also of those invertebrate such as the snails *L. stagnalis* and *D. magna* (Cardwell et al., 2018; Gensemer et al., 2018). Other species such as carp *C. carassius* (Poléo et al., 2017) and Atlantic salmon, *S. salar* (Jagoe and Haines, 1997), while managing to survive at high concentrations of Al, showed gill histopathological alterations. In other cases, instead, the Al exposure has led to abnormalities in reproduction (Yokel, 2020) and behaviour (Capriello et al., 2019, 2021a; Kumar and Gill, 2009). Its effects have also been studied in humans' beings, where the Al appears to contribute to the onset of neurodegenerative and neuromuscular diseases (Flaten, 1990) such as amyotrophic lateral sclerosis (Maya et al., 2018; Perl et al., 1982), Parkinson's (Bjorklund et al., 2018) and Alzheimer's disease (Colomina and Peris-Sampedro, 2017). However, despite these pieces of evidence, the mechanisms of action of the Al toxicity are not well understood compared with other metals such as cadmium (Favorito et al., 2010,2017; Ferrandino et al., 2009; Monaco et al., 2016), mercury (Zhang et al., 2016) or copper (Carotenuto et al., 2020), whose evident toxicity has been the subject of many studies. This work aimed to analyse the impact of the Al on the development of a known model organism, *Danio rerio,* better known as zebrafish, which is widely accepted in the scientific community as a suitable model organism for the ecotoxicological effects screening caused by environmental pollutants on vertebrates (Bambino and Chu, 2017; Favorito et al., 2011; Gerlai, 2010). Zebrafish embryos, especially, develop rapidly, and they are optically transparent, allowing making easy observations of any possible alterations in early life stages (Motta et al., 2019; Yang et al., 2009). In the present work, zebrafish embryos at 6 hours post-fertilization (hpf) were exposed to $AlCl_3$ for 72 hours (h) at concentrations of 50, 100 and 200 μM, corresponding respectively to 1.40, 2.80 and 5.60

mg/L of Al. These concentrations were chosen to simulate the conditions found in the polluted ground and surface waters (Agarwal et al., 2016, ATSDR, 2008), also taking into account the aluminium-dose effect on zebrafish larvae survival. Therefore, the Al concentrations used were all below the EC50 previously calculated and published (Monaco et al., 2017a). In earlier studies, we have already shown that the same Al concentrations induce behavioural changes in zebrafish larvae (Capriello et al., 2019). In this study, we further investigated the effects of Al exposure on zebrafish development. We evaluated the cell death response, given that it is a highly regulated and essential process for normal embryo development (Popgeorgiev et al., 2018), which may be affected by exposure to some substances, including metals (Monaco et al., 2017b; Zhao et al., 2020). Moreover, in this study, a special focus was placed on aspects related to oxidative stress as a potential mechanism of Al toxicity (Maheswari et al., 2014). It was assessed, especially, the involvement of reactive oxygen species (ROS) and different oxidative biomarkers such as antioxidants activity, oxidative damage and metallothioneins. The acetylcholinesterase activity was also evaluated because of its role in neuromuscular transmission. Moreover, in animals exposed to the highest concentration of the Al, we also analysed the expression pattern of genes involved in the neural development and function, c-*fos, app*a and *app*b, demonstrating the neurotoxic effect of the Al treatment. This research highlights that this metal is unsafe for the environment, and it lays the foundation for future work aiming to better define the effects of an as element used as dangerous.

2. Material and Methods

2.1 *Embryo growth*

Embryos were obtained from 15 healthy adult zebrafish (in ratio 2 female to 1 male) housed in oxygenated tanks with a natural photoperiod of 12 h:12 h light/dark, at a temperature of 28.5°C and a pH of 7.6. Adult zebrafish were fed twice a day with a commercial diet (TetraMin Tropical Flake Fish®) supplemented with *Artemia* sp. nauplii (Westerfield, 2000).

The eggs, laid at the first light on the morning, were collected by siphoning the bottom of the fish tank and housed in dishes containing E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2 \cdot 2H_2O$, 0.33 mM $MgSO_4$) in a water bath at 28.5 °C (Westerfield, 2000). After 6 hpf, using a stereomicroscope, unfertilized eggs (denser and whiter) were eliminated and the healthy embryos, which showed synchronous development, were selected for experimental procedures. All the experiments were performed according to the guidelines and policies dictated by European regulations on the wellness of animals employed for experimental purposes (Directive 2010/63/EU).

2.2 *Embryo treatments*

For Al treatments, a series of solutions were prepared at concentrations of 50, 100 and 200 µM starting from a stock solution of 8 mM $AlCl_3 \cdot 6H_2O$ (Carlo Erba, Italy; CAS no. 7784-13-6). Periodically, these concentrations have been evaluated and confirmed by MP-AES analysis (Microwave Plasma-Atomic Emission Spectrometry) conducted at the ACE-Analytical Chemistry for the Environment, Department of Chemical Sciences, University of Naples "Federico II", with the following Al mean values (mg/L of Al): Control < 0.001; 50 µM $AlCl_3$ = 1.40 ± 0.04; 100 µM $AlCl_3$ = 2.80 ± 0.09; 200 µM = 5.60 ± 0.23. Embryos at the shield stage (6 hpf) were randomly distributed into six-well plates (ten embryos per well) containing either 10 mL of Al solution or E3 medium for the control group. The E3 medium and the Al solutions were refreshed every day. Embryo development was monitored along all the treatment period, and only healthy larvae, without evident morphological anomalies, were included in the pool for subsequent biochemical and biomolecular analysis. This choice was made because a high number of malformed larvae could have altered the final results, thus making the data not comparable between the different groups, nor with the swimming data obtained previously, for which the same procedural selection was performed (Capriello et al., 2019). Four independent replicates containing 70 larvae at each concentration and 70 control larvae were used for biochemical biomarkers analysis and metallothioneins assay. qPCR was performed on three independent biological replicates of 70 larvae for the treated group (200 µM) and control group.

Moreover, 20 larvae for each experimental group were used for apoptosis analyses by acridine orange staining.

2.3 *Analysis of development and evaluation of apoptosis*

The embryo development was monitored under a Leica Zoom 2000 stereomicroscope, at a magnification of 10x and 20x; any phenotypic alterations were recorded at 24, 48 and 72 h after the beginning of the treatments. After 72 h of treatment (at 78 hpf) control and treated larvae were moved to a new multiwell to analyse the whole-mount apoptosis using acridine orange staining (Monaco et al., 2017b). Before proceeding with the staining, larvae were rinsed three times with breeding solution (E3 medium) to remove the residues of Al. Next, larvae were exposed at room temperature to a solution of acridine orange dye (5 μg/mL) for 30 min in the dark (Félix et al., 2017). After several rinses with E3 medium to remove the excess dye, the larvae were individually placed in a drop slide and the images were acquired by microscope (Zeiss, Germany) setting the FITC channel, that has the emission peak at 525 nm (green spectrum) and allows to visualise the apoptotic cells like fluorescent spots. Apoptotic cells were quantified by counting all fluorescent cells in the brain and along the tail using the cell count tool of ImageJ software (Image Analysis Software, Rasband, NIH). The average number of positive fluorescent cells observed in the treated was then related to the mean number of apoptotic cells found in the control organisms.

2.4 *Biochemical analyses*

2.4.1 Sample collection

For biochemical analysis, samples were collected after the 72 h exposure (at 78 hpf), washed in cold phosphate buffer saline (PBS), collected in 1.5 mL microfuge tubes and homogenized in cold buffer (0.32 mM of sucrose, 20 mM of HEPES, 1 mM of $MgCl_2$, and 0.5 mM of phenylmethyl sulfonylfluoride (PMSF) in ethanol, pH 7.4) as previously described in Félix et al., (2018). The homogenate was centrifuged at 4 °C at 15000 x g for 20 min, and the

supernatant was stored at –80 °C until analysis. For metallothioneins analysis, 70 larvae from each experimental group were inset in 1.5 mL microfuge tubes with buffer (0.5 M sucrose, 20 mM Tris-HCl buffer, pH 8.6, containing 0.01 % β-mercaptoethanol) and homogenized using a pellet mixer and cordless motor (VWR International, Carnaxide, Portugal). Entire homogenized samples were subsequently stored at –20 °C until analysis. Moreover, proteins determination was carried out with the BioTek's Take3™ Micro-Volume Plate by optical absorbance at 280 nm using bovine serum albumin (BSA) as a standard.

2.4.2 Determination of ROS accumulation and enzyme activities

All assays were performed at 30 °C and all experimental procedures were reported in detail in the Supplementary Material section. All samples were compared against a reagent blank and carried out in technical duplicate. ROS accumulation was evaluated by a previously reported methodology (Deng et al., 2009; Félix et al., 2018). Catalase (CAT) activity was evaluated according to the protocol reported by Claiborne (1985). The protocol reported in Durak et al., (1993) was used to determine the superoxide dismutase (SOD) activity. The activity of the glutathione S-transferases (GST) was assayed as reported by Habig and Jakoby, (1981) while the evaluation of Glutathione peroxidase (GPx) activity was carried out by spectrophotometric analysis at 340 nm as reported in Massarsky et al., (2017). ATPase activity was assayed by measuring the inorganic phosphate (Pi) released from ATP as described by Lança et al., (2015). Acetylcholinesterase (AChE) was analysed as reported in Rodriguez-Fuentes et al., (2015), while the activity of lactate dehydrogenase (LDH) was carried out as described by Domingues et al., (2010).

2.4.3 Determination of glutathione levels and oxidative stress index

The levels of reduced (GSH) and oxidized (GSSG) glutathione were tested as reported by Misra and Niyogi, (2009), with the fluorochrome ortho-phthalaldehyde (OPT) (1 mg/mL in methanol), in a Varian Cary Eclipse (Varian, USA) spectrofluorometer with a microplate

reader. See Supplementary Material section for detailed experimental procedures. The oxidative stress index (OSI) was calculated as the ratio between GSH (µmol of GSH/mg protein) and GSSG (µmol of GSH/mg protein).

2.4.4 Metallothioneins assay and oxidative damage biomarkers

The metallothioneins assay was performed as reported by Linde and Garcia-Vazquez, (2006). The lipid peroxidation (TBARS) was evaluated by the thiobarbituric (TBA) method (Wallin et al., 1993), while the oxidative damage to proteins (carbonyls, CO) was assayed by the DNPH (2,4-dinitrophenylhydrazine) according to the method described by Quintana-Villamandos et al., (2016). Detailed protocols were reported in the Supplementary Material section.

2.5 *RNA extraction and qPCR analysis*

To evaluate the effect of $AlCl_3$ stimulation on the *c-fos*, *app*a and *app*b gene expression, known markers of neuronal development and activity, the mRNA level was analysed using the RT-qPCR approach in $AlCl_3$ treated larvae at the highest concentration (200 µM) and control larvae. Total RNA from larvae was isolated using the TRI-Reagent (SIGMA-Aldrich®, St. Louis, MO, USA) according to the manufacturer's instructions. The concentration and purity of the RNA samples were assessed using a NanoDrop® 1000 spectrophotometer (Thermo Fisher, Waltham, MA, USA). First-strand cDNA was synthesized from 1 µg of total RNA in 20 µL of total volume reaction with SuperScript III reverse transcriptase (Invitrogen, Thermo Fisher). qPCR was performed on three independent biological replicates, in technical duplicate for each biological replicate using the SYBR green (Applied Biosystems, Foster City, CA, USA) method and an Applied Biosystems 7500 System. The reaction mixture contained 50 ng of cDNA template and 400 nM of each forward and reverse primer in a final volume of 15 µL. The primers employed for the expression pattern analysis were as follows: forward 5′-GTGCAGCACGGCTTCACCGA-3′ and reverse 5′-TTGAGCTGCGCCGTTGGAGG-3′ for *c-fos* gene (ENSDARG00000031683); forward 5′-CCTCGAGAGCTACCTGTCTG-3′ and

195 reverse 5′-CTCGGCCCGTACATACTTCT-3′ for *app*a gene (ENSDARG00000104279); forward 5′-
196 TAGAGGAGGTTGTGCGAGTG-3′ and reverse 5′-CTTCTGGAAGCGCATGTGTT-3′ for *app*b
197 gene (ENSDARG00000055543); forward 5′-CTGGAAAACAACCCAGCTCT-3′ and reverse 5′-
198 CGGACCTCAGTCAGATCCTC-3′ for *rplp0* gene (ENSDARG00000051783). The PCR conditions
199 included a denaturation step (95 °C for 10 min) followed by 40 cycles of amplification and
200 quantification (95 °C for 35 s, 60 °C for 1 min), followed by melt curve analysis to check the
201 amplification specificity. The relative gene expression levels were normalised to the *rplp0* reference
202 gene and calculated by the $2^{-\Delta\Delta Ct}$ method.

203 **2.6 *Statistical analysis***

204 A sample size calculation was carried out with the G*Power 3 (University of Düsseldorf, Germany)
205 based on standard deviations (Oliveira et al., 2013). The data were checked for normality and
206 homogeneity by Shapiro-Wilk and Brown-Forsythe test respectively, before evaluating the statistical
207 significance. For qPCR analysis, statistical significance was tested by using a two-tailed t-test with a
208 p-value cut-off of 0.05, while for all others was carried out a one-way analysis of variance ANOVA
209 followed by Tukey's pairwise comparison test. All data were processed using GraphPad-Prism 7
210 (GraphPad Software, La Jolla California USA, www.graphpad.com) and expressed as mean±SD. The
211 minimum level of acceptable significance was set at $p < 0.05$, and different lowercase letters were
212 used to indicate significant differences between groups.

213 **3. Results**

214 **3.1 *Phenotypic alterations***

215 The larvae exposed to $AlCl_3$ developed phenotypic alterations concerning head-tail axis
216 malformations, yolk sac swelling and pericardial edema (Fig. 1, panel I). Pericardial edema was
217 already visible at 24 h of the treatment, while the head-tail axis alteration was evident only after
218 hatching. After 72 h of exposure, the number of altered larvae increased with increasing $AlCl_3$

concentration (Fig. 1, panel II). Precisely, 13 ± 3 % ($p < 0.01$) and 23 ± 6 % ($p < 0.001$) of larvae showed malformations at the concentrations of 50 and 100 µM AlC_3, respectively. The percentage reached the value of 35 ± 9 % ($p < 0.001$) at the concentration of 200 µM. No teratogenic effects were observed in control larvae.

3.2 *Apoptosis induction*

Control and treated larvae with a normal phenotype were used to analyse the apoptosis in whole-mount by acridine orange staining. An increase in the number of fluorescent cells was observed in all treated larvae when compared to controls (Fig. 2). A physiological presence of apoptotic cells was visible in control larvae, both at the e head and the tail levels (Fig. 2A, E). A particular distribution of apoptotic cells was observed in the head of treated larvae, especially in larvae exposed to 50 (Fig. 2B) and 200 µM (Fig. 2D), in which apoptosis was localized in groups of cells of circumscribed area (indicated by arrows) unlike organisms treated with 100 µM (Fig. 2C). On the contrary, the distribution of the apoptotic cells in the tail was homogeneous for all the control and treated organisms (Fig. 2 E-H). The number of apoptotic cells in the head of larvae exposed to 50 µM is 8-fold higher compared to controls (Fig. 2, graph I). This number tended to decrease in larvae exposed to 100 ($p < 0.001$) and 200 µM ($p < 0.001$), although remaining significantly higher than the controls. Similarly, within the tail (Fig. 2, graph II), the number of apoptotic cells was about 10-fold higher for the larvae exposed to 50 µM ($p < 0.001$) compared to the controls and decreased slightly in larvae exposed to 100 ($p < 0.001$) and 200 µM ($p < 0.01$) while remaining significantly higher than that observed in the controls.

3.3 Biochemical changes

3.3.1 ROS accumulation and enzyme activities

The accumulation of ROS was expressed as µmol of DCF/mg of protein (Fig. 3A). The mean value was 47.70 ± 6.10 for the controls, and increased significantly for the larvae exposed to

50 μM $AlCl_3$ during 72 h (58.50 ± 10.40, $p < 0.05$). However, the average amount of ROS significantly decreased in larvae exposed to higher $AlCl_3$ concentrations, 100 (32.58 ± 3.85, $p < 0.01$) and 200 μM (34.57 ± 7.47, $p < 0.01$), respectively. The effects of exposure to $AlCl_3$ on the activities of the enzymes were variable, depending on the considered enzyme. The CAT activity, expressed as U/mg protein (Fig. 3B), and the LDH activity (Fig. 3C), expressed as nmol NADH/min·mg protein, showed no significant differences between control and treated groups. SOD and GST showed a similar trend, with a significant increase observed in their activity, in larvae exposed to 50 μM compared to control and larvae exposed to other concentrations. Precisely, the activity of SOD, expressed as U/mg protein (Fig. 3D), was twice for larvae exposed to 50 μM (31.72 ± 5.24, $p < 0.001$) compared to that measured in the controls (14.01 ± 3.33). For the concentrations of 100 (20.95 ± 4.05, $p < 0.05$) and 200 μM (18.52 ± 4.40, $p < 0.01$), the activity was higher compared to control larvae, but progressively decreased compared to larvae exposed to 50 μM. Similarly, the GST activity (Fig. 3E), expressed as nmol CDNB/min·mg protein, increased 1.6-fold (8.84 ± 1.86; $p < 0.05$) in the larvae exposed to the minimum concentration of $AlCl_3$ (50 μM) compared to the activity measured in control larvae (5.44 ± 0.43). At higher concentrations, GST activities (6.67 ± 1.35 for 100 μM; 7.39 ± 1.64 for 200 μM) tended to decrease reaching control values. Conversely, the GPx activity (Fig. 3F), expressed as nmol NADPH/min·mg protein, showed a concentration-dependent trend. The specimens exposed to 50 μM of $AlCl_3$ showed a lower GPx activity (1.20 ± 0.20, $p < 0.01$) than those of the controls (2.82 ± 0.13). The levels then significantly increased, reaching values of 1.74 ± 0.65 ($p < 0.05$) in larvae treated with 100 μM, and 2.33 ± 0.02 ($p < 0.05$) in those exposed to 200 μM. The ATPase activity was measured as mmol of Pi/mg of protein (Fig. 3G). The release of Pi significantly decreased for larvae treated with 50 μM of $AlCl_3$ with a mean value of 2.00 ± 0.11 ($p < 0.05$), compared to control animals (2.59 ± 0.40). Larvae exposed to other concentrations showed levels of Pi (2.15 ± 0.23 for 100 μM; 2.27 ± 0.11 for 200 μM) higher, with a trend reaching the value of

the controls. Acetylcholinesterase (AChE), a fundamental enzyme in neurotransmission, was expressed as nmol NADH/min·mg protein, showing a concentration-dependent increase trend (Fig. 3H). The levels of AChE in organisms exposed to 50 μM was 1.51 ± 0.29 compared to those of controls (0.98 ± 0.17), reaching 2.14 ± 0.55 ($p < 0.05$) and 2.23 ± 0.74 ($p < 0.05$) in organisms exposed to 100 and 200 μM $AlCl_3$, respectively.

3.3.2 Glutathione levels and oxidative stress index

The reduced glutathione levels (GSH), measured as μmol GSH/mg protein (Fig. 4A), for larvae treated with 50 μM $AlCl_3$ (0.13 ± 0.02, $p < 0.001$) were approximately half those of controls (0.27 ± 0.04) and tended to increase significantly for higher concentrations (0.18 ± 0.01, $p < 0.01$ for 100 μM and 0.18 ± 0.01, $p < 0.05$ for 200 μM). The oxidized glutathione levels (GSSG), expressed as μmol GSSG/mg protein (Fig. 4B), followed a similar trend to that of GSH, drastically decreasing for groups treated with 50 μM $AlCl_3$ (5.05 ± 1.33, $p < 0.001$), when compared to controls (22.59 ± 9.14) and tended to increase slightly at higher concentrations (7.40 ± 0.62, $p < 0.01$ for 100 μM and 5.98 ± 1.21, $p < 0.01$ for 200 μM). The oxidative stress index (OSI) also showed a similar trend to that of GSH and GSSG (Fig. 4C) with a significant decrease, comparing to controls (0.043 ± 0.002) in groups treated with 50 μM $AlCl_3$ (0.027 ± 0.004, $p < 0.05$) with a value that remained almost constant even for the highest concentrations (0.027 ± 0.007, $p < 0.05$ for 100 μM and 0.026 ± 0.003, $p < 0.05$ for 200 μM).

3.3.3 Metallothioneins assay and oxidative damage biomarkers

The levels of metallothioneins (MTs), expressed as nmol MTs/mg protein (Fig. 4D), significantly decreased in the larvae exposed to 50 μM $AlCl_3$ (14.23 ± 2.12, $p < 0.05$) compared to control (21.83 ± 2.91) and did not change in the organisms exposed to 100 μM (16.83 ± 1.67). Moreover, the levels were reduced by 4.6-fold (4.7 ± 3.44, $p < 0.001$) in larvae

293 treated with 200 μM compared to controls. Conversely, the trend of lipid peroxidation levels
294 (TBARS), measured as μmol MDA/mg protein (Fig. 4E), increased in a concentration-
295 dependent manner. The levels shown by control organisms (1.54 ± 0.74) were similar to that
296 shown by organisms exposed to 50 μM $AlCl_3$ (1.98 ± 0.77). Instead, it increased significantly
297 compared to controls in organisms exposed to 100 (4.6 ± 1.66, $p < 0.05$) and 200 μM (7.69 ±
298 1.89, $p < 0.001$). Otherwise, the oxidative damage of proteins (carbonyls, CO), expressed as
299 nmol NADPH/min·mg protein (Fig. 4F), fluctuated depending on the $AlCl_3$ concentration
300 tested. Indeed, the controls showed CO level (2.72 ± 0.54) similar to that of organisms exposed
301 to 100 μM (2.92 ± 0.30). The levels increased significantly for larvae exposed to the lowest
302 50 μM (4.04 ± 0.65, $p < 0.05$) and highest 200 μM (4.33 ± 0.92, $p < 0.05$) concentration.

303 ***3.4 Expression of c-fos, appa and appb genes***

304 Our results show a significantly higher transcript level of *c-fos* in $AlCl_3$-treated zebrafish larvae ($p <$
305 0.001) compared to control larvae (Figure 5). Our investigation was also extended to the zebrafish
306 *app*a and *app*b genes. Both the zebrafish *app* paralog genes showed a higher expression level in
307 $AlCl_3$-treated zebrafish larvae compared to control (Fig. 5), with a significant variation for *app*a gene
308 ($p < 0.05$).

309 **4. Discussion**

310 The contamination of water with aluminium may cause environmental pollution, becoming a crucial
311 health and survival problem for aquatic species (Ameri et al., 2020). In light of this, it is essential a
312 comprehensive analysis of its toxic effects. In our previous study, it has been shown that exposure to
313 Al induced changes in the swimming ability of larvae. In detail, compared to controls, the larvae
314 motility drastically decreased when exposed to 50 μM $AlCl_3$, but tended to increase if the larvae were
315 exposed to high concentrations (100 and 200 μM), without reaching the controls' performances
316 (Capriello et al., 2019). In this regard, it was evaluated under the same conditions, the morphology,
317 apoptosis, and the oxidative state of developing zebrafish to better understand the mode of action and

the toxic effects of this metal. The phenotypic alterations observed such as deviations of the axis and/or tail, swelling of the yolk sac or presence of pericardial edema, and their concentration-dependent increase are common evidence of toxic effects in zebrafish. These were also found after exposure to various agents such as metals (Jin et al., 2015; Xu et al., 2017), herbicides (Lanzarin et al., 2019) or dyes (Capriello et al., 2021b; Motta et al., 2019). The presence of phenotypic alterations manifested in developing zebrafish exposed to toxic substances was often associated with changes in apoptotic processes (Çomakli et al., 2018; Shi et al., 2008). Apoptosis is a process of programmed cell death, highly conserved and regulated, important in the morphogenesis of developing tissues and organs' homeostasis (Cole and Ross, 2001). Usually, during the development, the organisms produce excess cells that are removed by cell death (Jacobson et al., 1997). However, this biological mechanism may be altered following exposure to various substances, including metals such as cadmium (Monaco et al., 2017b) and copper (Zhao et al., 2020), thus hindering the optimal development of the organism. In this study, Al showed a high pro-apoptotic activity in zebrafish as already reported in mammalians by Mesole et al., (2020).

In particular, Al induced an increase in apoptosis at the level of the head and tail, especially in the organisms exposed to the lowest concentration (50 μM AlC_3), while those exposed to higher doses exhibited a decrease in the number of apoptotic cells. This is in agreement with the trend of the motility parameters previously evaluated (Capriello et al., 2019), in which larvae exposed to higher concentrations show better swimming performance than those exposed to lower ones. Thus, organisms with less damaged tissue structure probably showed better swimming activity.

A recent area of interest is the ability of Al to promote oxidative stress (Mujika et al., 2014). Indeed, the mammals showed changes in the oxidative state in reaction to Al exposure, in addition to the alteration of the apoptotic pathway (Mesole et al., 2020). This is surprising since Al is considered a non-redox metal but, as demonstrated by Kong et al., (1992), Al (III) can exert a significant pro-oxidant activity. Oxidative stress has become an important topic also in aquatic toxicity (Lushchak, 2011), as a possible mode of action for several pollutants, including metals (Adeyemi et al., 2015; Jin

et al., 2015). In this case, the amount of ROS produced increased in the organisms exposed to the lowest tested concentration of 50 μM $AlCl_3$ and decreased for the highest concentrations. This trend is comparable to the activities of some antioxidants evaluated, as SOD and GST, which act as a first antioxidant defence by reducing the free radical production, as already demonstrated in developing zebrafish after exposure to increasing concentrations of other metals such as cadmium and chromium (Jin et al., 2015). Moreover, Al may have both pro-oxidant and antioxidant activity, depending on the concentration (Oteiza et al., 1993), and this could explain the observed decrease in both ROS levels and antioxidant enzymes activity by increasing the concentration. The activity of enzyme GPx increased in a concentration-dependent manner, although remaining always below that recorded in the controls. A possible explanation for this discrepancy could be due, as reported by Exley (2004), to the possible stabilization by Al (III) of a superoxide radical anion $O_2^{\cdot -}$. This mechanism can induce the formation of several reactive oxygen species both by a direct pathway with the formation of the $^{\cdot}OOH$ radical and indirectly by influencing the redox equilibrium in the Fenton reaction. Therefore, the increase in GPx activity may not respond to a direct increase in ROS, like other enzymes (SOD and GST), but intervene subsequently to form secondary species. Furthermore, GPx plays a substantial role in protecting neural cells in response to extreme oxidative stress (Crack et al., 2001; Kishido et al., 2007). In this case, its protective action probably contributed either to a decrease in cell mortality (Seok et al., 2007) or an improvement in the swimming activity of the larvae (Strungaru et al., 2019), recorded at higher concentrations. The levels of GSH and its oxidized form (GSSG) were lower than those recorded in the controls and remained constant for all concentrations tested. This phenomenon is not uncommon in zebrafish in response to the induction of oxidative stress by metals such as arsenic (Adeyemi et al., 2015), cadmium or chromium (Jin et al., 2015), and in other aquatic species after exposure to mercury (Cappello et al., 2016). This was also observed in rats after exposure to Al (Singla and Dhawan, 2014). Moreover, this depletion of the intracellular pool of available GSH molecules is probably due to the high use of GSH as a substrate by various antioxidant enzymes (Massarsky et al., 2017). Indeed, GPX-catalyse the reduction of H_2O_2 at the expense of GSH

370 (Liu et al., 2008), while GST induce the formation of conjugates between the ion electrophilic and
371 GSH (Harvey, 2008; Singla and Dhawan, 2014), and these mechanisms are also associated with
372 concomitant oxidation of GSH to GSSG.
373 The MTs also play an essential role in the detoxification process acting as scavengers of toxic metal
374 ions or ROS (Chen et al., 2004). In this study, as for the other enzymes mentioned above, in the
375 organisms treated, the exposure to Al caused a response inversely proportional to the concentration,
376 with decreasing MTs levels with increasing concentration. For the specimens exposed to low
377 concentrations, the chelation of Al by MTs contributed to detoxification, attenuating the direct
378 production of ROS, reducing the absorption of metal ions and increasing their export from the cells
379 (Park et al., 2001). On the other hand, the levels of MTs drastically decreased after exposure to 200
380 μM $AlCl_3$ probably because the accumulation of Al could have reached a toxic threshold limit,
381 compromising the biosynthesis of MTs as reported by De Smet et al., (2001) and Wang et al., (2014)
382 in other aquatic organisms after exposure to other metals. Indeed, it is not uncommon that in fish, the
383 transcription and translation of MTs are compromised by exposure to a pollutant (Rhee et al., 2009),
384 this would also explain the discrepancy in the levels of MTs, higher in the controls, compared to the
385 treated animals. Overall, the decrease, either in cell mortality or in ROS and antioxidant production
386 at maximum concentration, agrees with the improvement in swimming performance previously
387 recorded. This phenomenon might seem to oppose the usual toxic concentration-response effects, but
388 it has already been described several times in zebrafish after acute or prolonged exposure to metals
389 (Avallone et al., 2015; Bui Thi et al., 2020; Jijie et al., 2020; Pilehvar et al., 2020). The decrease of
390 toxic effects at high or prolonged exposures is due to the activation of an adaptive response that allows
391 it to increase the resistance to cellular stress and survive even in less favourable conditions (Messerli
392 et al., 2020). However, despite the activation of antioxidant defence mechanisms, oxidative damage
393 on cell components was evident. It is known that Al cannot initiate peroxidation, but it can attack the
394 cell components, including membrane lipids (Flora et al., 2003). In this study, the lipid peroxidation
395 (TBARS) increased with the increase of Al concentration, probably due to the inactivation of some

antioxidant enzymes derived to conformational changes induced by Al and/or the oxidative modification of genes that control these enzymes. This phenomenon is also reported in the other studies where similar effects were observed in mammals exposed to Al (Abdel Moneim et al., 2012; Nehru and Anand, 2005; Youself, 2004). On the other hand, the oxidative damage to proteins acted in the same direction, albeit in a less linear way, probably because carbonyl proteins (CO) are more stable than TBARS (Bizzozero, 2009). In particular, a decrease in CO levels was evident in the organisms exposed to the concentration of 100 μM $AlCl_3$ compared to those exposed to the other two concentrations (50 and 200 μM). A similar phenomenon, where an intermediate exposure led to a decrease in CO levels with subsequent recovery was already observed in zebrafish after acute cold exposure (Tseng et al., 2011). However, further studies are needed to understand the mechanism behind the basis of this phenomenon. The metabolic activity assessed through the analysis of ATPase activity reflected a slight decrease only for the organisms exposed to 50 μM $AlCl_3$, while for the concentration of 100 and 200 μM, the activity was similar to that recorded in the controls. ATPase is involved in energy metabolism (Abad, 2011) and its activity, which tended to return to physiological levels in larvae exposed to higher concentrations, corroborates the previously evaluated motility data (Capriello et al., 2019). Instead, the activity of acetylcholinesterase (AChE) increased linearly with the concentration. AChE is the enzyme that catalyses the breakdown of acetylcholine (ACh) in the neural synapse, and its activity can be positively affected by environmental contaminants such as heavy metals, as reported by Bui Thi et al., (2020). In particular, Al can interact with the cholinergic system like a cholinotoxin (Gulya et al., 1990), increasing acetylcholinesterase activity in zebrafish (Senger et al., 2011). It is also interesting to note the correlation between the trend of this enzyme and the concentration-dependent increase in the number of unchecked movements carried out by the larvae, evaluated in the previous study (Capriello et al., 2019). Cholinergic systems are involved in locomotor activity responses and movements regulation (Hagino et al., 2015). Indeed, new drugs, as cholinesterase inhibitors, are used to block the symptoms of tremor or dyskinesias typical of Parkinson's disease (Korczyn, 2004).

Even though Al seemed to induce fewer effects at the maximum concentration, we revealed an induced alteration in the gene expression level of *c-fos, app*a and *app*b, known markers of neuronal development and function. *c-fos* gene showed higher expression levels compared to the controls. *c-fos* is considered an immediate-early gene whose expression increases after neuronal activation (Stewart et al., 2014). Indeed, an increase in its expression was also detected in zebrafish after exposure to various toxic substances (Lefebvre et al., 2009; Özdemir et al., 2018) and recently reported also in a prenatal stress paradigm in the zebrafish larvae (D'Agostino et al., 2019). Moreover, it is known that its expression is also induced in cells when apoptosis begins (Vyas et al., 2002). For this reason, in the present study, the increase in the expression of the *c-fos* could be associated with apoptosis induction by Al, as occurred in zebrafish larvae exposed to other toxic substances (Çomakli et al., 2018). *app*a and *app*b genes, the two homologs of the mammalian amyloid precursor protein (APP) are essential genes in neurodevelopment and the formation of normal locomotor behaviour, both in mammals (Sosa et al., 2017) and zebrafish (Abramsson et al., 2013; Kaiser et al., 2012). Moreover, the up-regulated expression of *app* occurs early in the cascade of events that leads to amyloid plaque formation, typical of the Alzheimer's disease pathogenesis (Andrew et al., 2016; Walton and Wang, 2009; Zhang et al., 2011). In this study, only *app*a showed a significant variation of expression levels in the treated organisms compared to the controls. This increase is corroborated with other studies showing that *app* gene expression is significantly up-regulated in human and rat neural cells, in response to stress induced by Al ions (Lukiw et al., 2005; Walton and Wang, 2009). The different expression between *app*a and *app*b could be linked to the stage of development, as demonstrated by Musa et al., (2001). In fact, their levels change during the development of zebrafish, altering according to a compensatory mechanism (Banote et al., 2020).

Conclusion

Our study proved that exposure to Al may affect a range of biological processes that impact zebrafish development. Exposure to increasing concentrations of AlC_3 for 72 h induced concentration-

dependent teratogenic effects, while apoptosis and oxidative stress seemed to decline at high concentrations. In detail, the involvement of ROS and some antioxidant enzymes such as SOD and GST and of MTs, whose role in zebrafish is to prevent or reduce the effects of ROS, were reduced at maximum concentration also in agreement with data relating to metabolism (ATPase) and neuromuscular transmission (AChE) that showed the same trend. However, the maximum concentration was far from harmless for the zebrafish larvae, which presented altered expression of neural development and function genes. These results contribute to elucidate the effects of Al on developing zebrafish. Still, it should be pointed out that little information is available on the action mechanism of this metal and further investigation is necessary to clarify all possible implications on the environment due to its growing diffusion.

Conflict of interest

The authors declare that there is no potential conflict of interest concerning the authorship, research, and/or finances.

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References

Abad, J.P., 2011. ATPase. In: Gargaud M. et al. (eds) Encyclopedia of Astrobiology. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-11274-4_135.

Abdel Moneim, A.E., 2012. Evaluating the potential role of pomegranate peel in aluminum-induced oxidative stress and histopathological alterations in brain of female rats. Biol. Trace Elem. Res.; 150(1-3),328–336. DOI:10.1007/s12011-012-9498-2.

Abramsson, A., Kettunen, P., Banote, R.K., Lott, E., Li, M., Arner, A., Zetterberg, H., 2013. The zebrafish amyloid precursor protein-b is required for motor neuron guidance and synapse formation. Dev. Biol.; 381(2):377-88. DOI: 10.1016/j.ydbio.2013.06.026.

Adams, W.J., Cardwell, A.S., DeForest, D.K., Gensemer, R.W., Santorec, R.C., Wang, N., Nordheim, E., 2018. Aluminum bioavailability and toxicity to aquatic organisms: Introduction to the special section. Environ. Toxicol. Chem.; 37(1):34-35. DOI: 10.1002/etc.3879.

Adeyemi, J.A., da Cunha Martins-Junior, A., Barbosa, F. Jr., 2015. Teratogenicity, genotoxicity and oxidative stress in zebrafish embryos (Danio rerio) co-exposed to arsenic and atrazine. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 172-173,7–12. DOI:10.1016/j.cbpc.2015.04.001.

Agarwal, S., Tyagi, I., Gupta,V.K., Dehghani, M.H., Ghanbari, R., 2016. Investigating the residual aluminum elimination from conventional and enhanced coagulation by phosphate compounds in wastewater treatment process. J. Mol. Liq.; 221:673-684.https://doi.org/10.1016/j.molliq.2016.06.051.

Agency for Toxic Substances and Disease (ATSDR), 2008. Toxicological Profile for Aluminum. CAS: 7429-90-5.

Ameri, M., Baron-Sola, A., Khavari-Nejad, R.A., Soltani, N., Najafi, F., Bagheri, A., Martinez, F., Hernández, L.E., 2020. Aluminium triggers oxidative stress and antioxidant response in the microalgae Scenedesmus sp. J. Plant Physiol.; 246-247:153114. DOI: 10.1016/j.jplph.2020.153114.

Andrew, R.J., Kellett, K.A., Thinakaran, G., Hooper, N.M., 2016. A Greek Tragedy: The Growing Complexity of Alzheimer Amyloid Precursor Protein Proteolysis. J. Biol. Chem.; 291(37):19235-44. DOI: 10.1074/jbc.R116.746032.

Avallone, B., Crispino, R., Cerciello, R., Simoniello, P., Panzuto, R., Motta, M.C., 2015. Cadmium effects on the retina of adult Danio rerio. C. R. Biol.; 338(1):40-7. DOI: 10.1016/j.crvi.2014.10.005.

495 Bambino, K., Chu, J., 2017. Zebrafish in Toxicology and Environmental Health. Curr. Top Dev. Biol.;
496 124:331-367. DOI: 10.1016/bs.ctdb.2016.10.007.

497 Banote, R.K., Chebli, J., Şatır, T.M., Varshney, G.K., Camacho, R., Ledin, J., Burgess, S.M., Abramsson, A.,
498 Zetterberg, H., 2020. Amyloid precursor protein-b facilitates cell adhesion during early development in
499 zebrafish. Sci. Rep.; 10(1):10127. DOI: 10.1038/s41598-020-66584-8.

500 Barabasz, W., Albinska, D., Jaskowska, M., Lipiec, J., 2002. Ecotoxicology of aluminium. Pol. J. Environ.
501 Stud. 11 (3), 199e203.

502 Bizzozero, O. A., 2009. Protein Carbonylation in Neurodegenerative and Demyelinating CNS Diseases.
503 Handbook of Neurochemistry and Molecular Neurobiology (Springer US); 543-562. DOI: 10.1007/978-0-387-
504 30375-8_23.

505 Bjorklund, G., Stejskal, V., Urbina, M.A., Dadar, M., Chirumbolo, S., Mutter, J., 2018. Metals and Parkinson's
506 Disease: Mechanisms and Biochemical Processes. Curr. Med. Chem.; 25(19):2198-2214. DOI:
507 10.2174/0929867325666171129124616.

508 Bui Thi, N.H., Nguyen Thi, N.A., Audira, G., Siregar, P., Liang, S.T., Huang, J.C., Hsiao, C.D., 2020. Chronic
509 Exposure to Low Concentration Lead Chloride-Induced Anxiety and Loss of Aggression and Memory in
510 Zebrafish. Int. J. Mol. Sci., 7, 21(5). DOI: 10.3390/ijms21051844.

511 Cappello, T., Brandão, F., Guilherme, S., Santos, M.A., Maisano, M., Mauceri, A., Canário, J., Pacheco, M.,
512 Pereira, P., 2016. Insights into the mechanisms underlying mercury-induced oxidative stress in gills of wild
513 fish (Liza aurata) combining (1)H NMR metabolomics and conventional biochemical assays. Sci. Total.
514 Environ.; 548-549,13–24. DOI:10.1016/j.scitotenv.2016.01.008.

515 Capriello, T., Félix, L.M., Monteiro, S.M., Santos, D., Cofone, R., Ferrandino, I., 2021a. Exposure to
516 aluminium causes behavioural alterations and oxidative stress in the brain of adult zebrafish. Environ. Toxicol.
517 Pharmacol.; 85:103636. DOI: 10.1016/j.etap.2021.103636.

518 Capriello, T., Grimaldi, M.C., Cofone, R., D'Aniello, S., Ferrandino, I., 2019. Effects of aluminium and
519 cadmium on hatching and swimming ability in developing zebrafish. Chemosphere; 222:243-249. DOI:
520 10.1016/j.chemosphere.2019.01.140.

521 Capriello, T., Visone, I.M., Motta, C.M., Ferrandino, I., 2021b. Adverse effects of E150d on zebrafish
522 development. Food Chem. Toxicol; 147:111877. DOI: 10.1016/j.fct.2020.111877.

523 Cardwell, A.S., Adams, W.J., Gensemer, R.W., Nordheim, E., Santore, R.C., Ryan, A.C., Stubblefield, W.A.,
524 2018. Chronic toxicity of aluminum, at a pH of 6, tofreshwater organisms: Empirical data for the development
525 of interna-tional regulatory standards/criteria. Environ. Toxicol. Chem.; 37:36–48. DOI:10.1002/etc.3901.

526 Carotenuto, R., Capriello, T., Cofone, R., Galdiero, G., Fogliano, C., Ferrandino, I., 2020. Impact of copper in
527 Xenopus laevis liver: Histological damages and atp7b downregulation. Ecotoxicol. Environ. Saf.; 30,188-
528 109940. https://doi.org/10.1016/j.ecoenv.2019.109940.

529 Chen, W.Y., John, J.A.C., Lin, C-H., Lin, H.-F., Wu, S.-C., Lin, C.-H., Chang, C-Y., 2004. Expression of
530 Metallothionein gene during embryonic and early larval development in zebrafish. Aquat. Toxicol.; 69,215–
531 227.DOI: 10.1016/j.aquatox.2004.05.004.

532 Claiborne, A., 1985.Catalase activity. Catalase activity, 283-84.

533 Cole, L.K., Ross, L.S., 2001. Apoptosis in the developing zebrafish embryo. Dev. Biol.; 240(1):123-42. DOI:
534 10.1006/dbio.2001.0432.

535 Coleman, J.G., Johnson, D.R., Stanley, J.K. Bednar, A.J., Weiss Jr. C.A, Boyd R.E., Steevens, J.A., 2010.
536 Assessing the fate and effects of nano aluminum oxide in the terrestrial earthworm, Eisenia fetida. Environ
537 Toxicol Chem. 2010;29(7):1575-1580. doi:10.1002/etc.196.

538 Colomina, M.T., Peris-Sampedro, F., 2017. Aluminum and Alzheimer's Disease. Adv Neurobiol.; 18:183-197.
539 DOI: 10.1007/978-3-319-60189-2_9.

540 Çomakli, S., Köktürk, M., Topal, A., Özkaraca, M., Ceyhun, S.B. ,2018. Immunofluorescence/fluorescence
541 assessment of brain-derived neurotrophic factor, c-Fos activation, and apoptosis in the brain of zebrafish
542 (Danio rerio) larvae exposed to glufosinate. Neurotoxicology.; 69:60-67. DOI: 10.1016/j.neuro.2018.09.003.

543 Crack, P.J., Taylor, J.M., Flentjar, N.J., de Haan, J., Hertzog, P., Iannello, R.C., Kola, I., 2001. Increased infarct
544 size and exacerbated apoptosis in the glutathione peroxidase-1 (Gpx-1) knockout mouse brain in response to
545 ischemia/reperfusion injury. J. Neurochem.; 78(6):1389-99. DOI: 10.1046/j.1471-4159.2001.00535.x. PMID:
546 11579147.

547 D'Agostino, S., Testa, M., Aliperti, V., Venditti, M., Minucci, S., Aniello, F., Donizetti, A., 2019. Expression
548 pattern dysregulation of stress- and neuronal activity-related genes in response to prenatal stress paradigm in
549 zebrafish larvae. Cell Stress Chaperones; 24(5):1005-1012. DOI: 10.1007/s12192-019-01017-8.

550 De Smet, H., De Wachter, B., Lobinski, R., Blust, R., 2001. Dynamics of (Cd,Zn)-metallothioneins in gills,
551 liver and kidney of common carp Cyprinus carpio during cadmium exposure. Aquat. Toxicol.; 52, 269–
552 281.DOI: 10.1016/S0166-445X(00)00136-3.

553 Deng, J., Yu, L., Liu, C., Yu, K., Shi, X., Yeung, L.W., Lam, P.K., Wu, R.S., Zhou, B., 2009.
554 Hexabromocyclododecane-induced developmental toxicity and apoptosis in zebrafish embryos. Aquat.
555 Toxicol.; 29-36. DOI: 10.1016/j.aquatox.2018.03.017.

556 Domingues, I., Oliveira, R., Lourenco, J., Grisolia, C.K., Mendo, S., Soares, A.M., 2010. Biomarkers as a tool
557 to assess effects of chromium (VI): comparison of responses in zebrafish early life stages and adults. Comp.
558 Biochem. Physiol. C Toxicol. Pharmacol. 152, 338-45. DOI: 10.1016/j.cbpc.2010.05.010.

559 Durak, I., Yurtarslanl, Z., Canbolat, O., Akyol, O., 1993. A methodological approach to superoxide dismutase
560 (SOD) activity assay based on inhibition of nitroblue tetrazolium (NBT) reduction. Clin. Chim. Acta.; 214,
561 103-4. DOI: 10.1016/0009-8981(93)90307-p.

562 Exley, C., 2004. The pro-oxidant activity of aluminum. Free Radic. Biol. Med.; 36(3):380-7. DOI:
563 10.1016/j.freeradbiomed.2003.11.017.

564 Favorito, R., Chiarelli, G., Grimaldi, M.C., De Bonis, S., Lancieri, M., Ferrandino, I., 2011. Bioaccumulation
565 of cadmium and its cytotoxic effect on zebrafish brain. Chem. Ecol.; 27(2),39-46.
566 https://doi.org/10.1080/02757540.2011.625937.

567 Favorito, R., Grimaldi, M.C., Coppola, M., Ferrandino, I., 2010. Effects of acute cadmium exposure on the
568 pituitary gland of Podarcis sicula. Open. Zool. J.; 3,30-6. DOI:10.2174/1874336601003010030.

569 Favorito, R., Monaco, A., Grimaldi, M.C., Ferrandino, I., 2017. Effects of cadmium on the glial architecture
570 in lizard brain. Eur. J. Histochem.; 61,2734. DOI: 10.4081/ejh.2017.2734.

571 Félix, L.M., Serafim, C., Valentim, A.M., Antunes, L.M., Matos, M., Coimbra, A.M., 2017. Apoptosis-related
572 genes induced in response to ketamine during early life stages of zebrafish. Toxicol Lett.; 279:1-8. DOI:
573 10.1016/j.toxlet.2017.07.888.

574 Félix, L.M., Vidal, A.M., Serafim, C., Valentim, A.M. Antunes, L.M., Monteiro, S.M., Matos, M., Ana M
575 Coimbra, A.M., 2018. Ketamine induction of p53-dependent apoptosis and oxidative stress in zebrafish (Danio
576 rerio) embryos. Chemosphere, 201,730–739. DOI:10.1016/j.chemosphere.2018.03.049.

577 Ferrandino, I., Favorito, R., Annunziata, M., Grimaldi, M.C., 2009. Cadmium induces apoptosis in the pituitary
578 gland of Podarcis sicula. Ann. N. Y. Acad. Sci.; 1163,386-8. DOI:10.1111/j.1749-6632.2008.03667.x.

579 Flaten, T.P., 1990. Geographical associations between aluminium in drinking water and death rates with
580 dementia (including Alzheimer's disease), Parkinson's disease and amyotrophic lateral sclerosis in Norway.
581 Environ. Geochem. Health 12 (1e2), 152e167. https://doi.org/10.1007/BF01734064.

582 Flora, S.J., Mehta, A., Satsangi, K., Kannan, G.M., Gupta, M., 2003. Aluminum-induced oxidative stress in
583 rat brain: response to combined administration of citric acid and HEDTA. Comp. Biochem. Physiol. Part C;
584 134,19–328. DOI: 10.1016/s1532-0456(02)00269-7.

585 Gensemer, R.W., Gondek, J.C., Rodriquez, P.H., Arbildua, J.J., Stubblefield, W.A., Cardwell, A.S., Santore,
586 R.C., Ryan, A.C., Adams, W.J., Nordheim, E., 2018. Evaluating the effects of pH, hardness, and dissolved
587 organic carbon on the toxicity of aluminum to freshwater aquatic organisms under circumneutral conditions.
588 Environ. Toxicol. Chem.; 37(1):49-60. DOI: 10.1002/etc.3920.

589 Gerlai, R., 2010. High-throughput behavioral screens: the first step towards finding genes involved in
590 vertebrate brain function using zebrafish. Molecules; 15(4):2609-22. DOI: 10.3390/molecules15042609.

591 Gulya, K., Rakonczay, Z., Kása, P., 1990. Cholinotoxic effects of aluminum in rat brain J Neurochem.; 54(3),
592 1020–1026. DOI:10.1111/j.1471-4159.1990.tb02352.x.

593 Habig, W.H., Jakoby, W.B., 1981. Assays for differentiation of glutathione S-Transferases. Methods Enzymol;
594 77,398-405. DOI: 10.1016/s0076-6879(81)77053-8.

595 Hagino, Y., Kasai, S., Fujita, M., Setogawa, S., Yamaura, H., Yanagihara, D., Hashimoto, M., Kobayashi K.,
596 Meltzer, H.Y., Ikeda, K.,2015. Involvement of cholinergic system in hyperactivity in dopamine-deficient mice.
597 Neuropsychopharmacology; 40(5):1141-50. DOI: 10.1038/npp.2014.295.

598 Harvey, J.W., 2008. Chapter 7- The Erythrocyte: Physiology, Metabolism, and Biochemical Disorders.
599 Clinical Biochemistry of Domestic Animals (Sixth Edition); 173-240. https://doi.org/10.1016/B978-0-12-
600 370491-7.00007-6.

601 Jacobson, M.D., Weil, M., Raff, M.C., 1997. Programmed cell death in animal development. Cell; 88(3):347-
602 54. DOI: 10.1016/s0092-8674(00)81873-5.

603 Jagoe, C.H., Haines, T.A., 1997. Changes in gill morphology of Atlantic salmon (Salmo salar) smolts due to
604 addition of acid and aluminum to stream water. Environ. Pollut.; 97(1-2):137-46. DOI: 10.1016/s0269-
605 7491(97)00069-9.

606 Jijie, R., Solcan, G., Nicoara, M.,Micu, D., Strungaru, S., 2020. Antagonistic Effects in Zebrafish (Danio
607 Rerio) Behavior and Oxidative Stress Induced by Toxic Metals and Deltamethrin Acute Exposure. Sci. Total.
608 Environ.; 698, 134299. DOI: 10.1016/j.scitotenv.2019.134299.

609 Jin, Y., Liu, Z., Liu, F., Ye, Y., Peng, T., Fu, Z., 2015. Embryonic exposure to cadmium (II) and chromium
610 (VI) induce behavioral alterations, oxidative stress and immunotoxicity in zebrafish (Danio rerio).
611 Neurotoxicol Teratol.; 48:9-17. DOI: 10.1016/j.ntt.2015.01.002.

612 Kaiser, D.M., Acharya, M., Leighton, P.L.A., Wang, H., Daude, N., Wohlgemuth, S., Shi, B., Allison, W.T,
613 2012. Amyloid beta precursor protein and prion protein have a conserved interaction affecting cell adhesion
614 and CNS development. PLoS One; 7(12):e51305. DOI: 10.1371/journal.pone.0051305.

615 Kishido, T., Unno, K., Yoshida, H., Choba, D., Fukutomi, R., Asahina, S., Iguchi, K., Oku, N., Hoshino,
616 M., 2007. Decline in glutathione peroxidase activity is a reason for brain senescence: consumption of green
617 tea catechin prevents the decline in its activity and protein oxidative damage in ageing mouse brain.
618 Biogerontology, 8(4),423-430. DOI:10.1007/s10522-007-9085-7.

619 Kong, S., Liochev, S., Fridovich, I., 1992. Aluminum(III) facilitates the oxidation of NADH by the superoxide
620 anion. Free Radic. Biol. Med.; 13(1):79-81. DOI: 10.1016/0891-5849(92)90168-g.

621 Korczyn, A.D., 2004. Drug treatment of Parkinson's disease. Dialogues Clin. Neurosci.; 6(3):315-22. DOI:
622 10.31887/DCNS.2004.6.3/akorczyn.

623 Kumar, V., Gill, K.D., 2009. Aluminium neurotoxicity: neurobehavioural and oxidative aspects. Arch.
624 Toxicol.; 83: 965–978. https://doi.org/10.1007/s00204-009-0455-6.

625 Lança, M.J., Machado, M., Ferreira, A.F., Quintella, B.R., de Almeida P.R., 2015. Structural lipid changes and
626 Na(+)/K(+)-ATPase activity of gill cells' basolateral membranes during saltwater acclimation in sea lamprey
627 (Petromyzon marinus, L.) juveniles. Comp. Biochem. Physiol. A Mol. Integr. Physiol.; 189,67-75. DOI:
628 10.1016/j.cbpa.2015.07.018.

629 Lanzarin, G.A.B., Félix, L.M., Santos, D., Venâncio, C.A.S., Monteiro, S.M., 2019. Dose-dependent effects
630 of a glyphosate commercial formulation-Roundup® UltraMax - on the early zebrafish embryogenesis.
631 Chemosphere, 223,514–522. DOI:10.1016/j.chemosphere.2019.02.071.

632 Lawrence, G.B., Sutherland, J. W., Boylen, C. W., Nierzwicki-Bauer, S. W., Momen, B., Baldigo, B. P.,
633 Simonin, H.A., 2007. Acid Rain Effects on Aluminum Mobilization Clarified by Inclusion of Strong Organic
634 Acids. Environ. Sci. Technol.; 41, 1, 93-98. https://doi.org/10.1021/es061437v.

635 Lefebvre, K.A., Tilton, S.C., Bammler, T.K., Beyer, R.P., Srinouanprachan, S., Stapleton, P.L., Farin, F.M.,
636 Gallagher, E.P., 2009. Gene expression profiles in zebrafish brain after acute exposure to domoic acid at
637 symptomatic and asymptomatic doses. Toxicol Sci.; 107(1):65-77. DOI: 10.1093/toxsci/kfn207.

638 Linde, A.R, Garcia-Vazquez, E., 2006. A Simple Assay to Quantify Metallothionein Helps to Learn About
639 Bioindicators and Environmental Health. Biochem. Mol. Biol. Educ.; 34 (5), 360-3. DOI:
640 10.1002/bmb.2006.494034052653.

641 Liu, Y., Wang, J., Wei, Y., Zhang, H., Xu, M., Dai, J., 2008. Induction of time-dependent oxidative stress and
642 related transcriptional effects of perfluorododecanoic acid in zebrafish liver. Aquat. Toxicol.; 89(4):242-50.
643 DOI: 10.1016/j.aquatox.2008.07.009.

644 Lukiw, W.J., Percy, M.E., Kruck, T.P., 2005. Nanomolar aluminum induces pro-inflammatory and pro-
645 apoptotic gene expression in human brain cells in primary culture. J. Inorg. Biochem.; 99(9):1895-8. doi:
646 10.1016/j.jinorgbio.2005.04.021.

647 Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol.;
648 101(1):13-30. DOI: 10.1016/j.aquatox.2010.10.006.

649 Maheswari, S.L., Venkatakrishna Murali, R., Balaji, R., 2014. Aluminium induced cholinotoxicity in zebra
650 fish brain - A sequel of oxidative stress. IJAR; 2(2):322-335.

651 Massarsky, A., Kozal, J.S., Di Giulio, R.T., 2017.Glutathione and zebrafish: Old assays to address a current
652 issue. Chemosphere, 168, 707-15. DOI: 10.1016/j.chemosphere.2016.11.004.

653 Maya, S., Prakash, T., Goli, D., 2018. Evaluation of neuroprotective effects of wedelolactone and gallic acid
654 on aluminium-induced neurodegeneration: Relevance to sporadic amyotrophic lateral sclerosis. Eur J
655 Pharmacol., 15;835:41-51. DOI: 10.1016/j.ejphar.2018.07.058.

656 Mesole, S.B., Alfred, O.O., Yusuf, U.A., Lukubi, L., Ndhlovu, D., 2020. Apoptotic Inducement of Neuronal
657 Cells by Aluminium Chloride and the Neuroprotective Effect of Eugenol in Wistar Rats. Oxid. Med. Cell.
658 Longev.; 8425643. DOI: 10.1155/2020/8425643.

659 Messerli, M., Aaldijk, D., Haberthür, D., Röss, H., García-Poyatos, C., Sande-Melón, M., Khoma, O.Z.,
660 Wieland, F.A.M., Fark, S., Djonov, V., 2020. Adaptation mechanism of the adult zebrafish respiratory organ
661 to endurance training. PLoS One; 15(2):e0228333. DOI: 10.1371/journal.pone.0228333.

662 Misra, S., Niyogi, S., 2009. Selenite causes cytotoxicity in rainbow trout (Oncorhynchus mykiss) hepatocytes
663 by inducing oxidative stress. Toxicol. In Vitro; 23, 1249-58. DOI: 10.1016/j.tiv.2009.07.03.

664 Monaco, A., Capriello, T., Grimaldi, M.C., Schiano, V., Ferrandino I., 2017b. Neurodegeneration in zebrafish
665 embryos and adults after cadmium exposure. Journal List. Eur. J. Histochem.; 61(4); 19PMC5656802. DOI:
666 10.4081/ejh.2017.2833.

667 Monaco, A., Grimaldi, M.C., Ferrandino, I., 2016. Neuroglial alterations in the zebrafish brain exposed to
668 cadmium chloride. J. Appl. Toxicol.; 36(12),1629-38. DOI: 10.1002/jat.3328.

669 Monaco, A., Grimaldi, M.C., Ferrandino, I., 2017a. Aluminium chloride-induced toxicity in zebrafish larvae.
670 J. Fish. Dis.; 40(5), 629-35. DOI: 10.1111/jfd.12544.

671 Motta, C.M., Simoniello, P., Arena, C., Capriello, T., Panzuto, R., Vitale, E., Agnisola, C., Tizzano, M.,
672 Avallone, B., Ferrandino, I., 2019. Effects of four food dyes on development of three model species, Cucumis
673 sativus, Artemia salina and Danio rerio: assessment of potential risk for the environment. Environ. Pollut.;
674 253,1126-1135. https://doi.org/10.1016/j.envpol.2019.06.018.

675 Mujika, J.I., Rezabal, E., Mercero, J.M., Ruipérez, F., Costa, D., Ugalde, J.M., Lopez, X., 2014. Aluminium
676 in biological environments: a computational approach. Comput. Struct. Biotechnol. J.; 9:e201403002. DOI:
677 10.5936/csbj.201403002.

678 Musa, A., Lehrach, H., Russo, V.A., 2001. Distinct expression patterns of two zebrafish homologues of the
679 human APP gene during embryonic development. Dev. Genes Evol.; 211(11):563-7. DOI: 10.1007/s00427-
680 001-0189-9.

681 Nehru, B., Anand, P., 2005. Oxidative damage following chronic aluminium exposure in adult and pup rat
682 brains. J. Trace Elem. Med. Biol.; 19(2-3):203-8. DOI: 10.1016/j.jtemb.2005.09.004.

683 Oliveira, M., Teixeira, J.C., Vasconcelos-Nobrega, C., Félix, L.M., Sardao, V.A., Colaco, A.A., Oliveira, P.A.,
684 Peixoto, F.P., 2013. Mitochondrial and liver oxidative stress alterations induced by N-butyl-N-(4-
685 hydroxybutyl) nitrosamine: relevance for hepatotoxicity. J. Appl. Toxicol.; 33, 434–443.
686 https://doi.org/10.1002/jat.1763.

687 Oteiza, P.I., Fraga, C.G., Keen, C.L., 1993. Aluminum has both oxidant and antioxidant effects in mouse brain
688 membranes. Arch. Biochem. Biophys.; 300(1):517-21. DOI: 10.1006/abbi.1993.1070.

689 Özdemir, S., Altun, S., Özkaraca, M., Ghosi, A., Toraman, E., Arslan, H., 2018. Cypermethrin, chlorpyrifos,
690 deltamethrin, and imidacloprid exposure up-regulates the mRNA and protein levels of bdnf and c-fos in the
691 brain of adult zebrafish (Danio rerio). Chemosphere; 203:318-326. DOI: 10.1016/j.chemosphere.2018.03.190.

692 Park, J.D., Liu, Y., Klaassen, C.D., 2001. Protective effect of metallothionein against the toxicity of cadmium
693 and other metals(1). Toxicology, 163(2-3),93–100. DOI:10.1016/s0300-483x(01)00375-4.

694 Pereira, S., Cavalie, I., Camilleri, V., Gilbin, R., Adam-Guillermin, C., 2013. Comparative genotoxicity of
695 aluminium and cadmium in embryonic zebrafish cells. Mutat Res. 20;750(1-2):19-26. DOI:
696 10.1016/j.mrgentox.2012.07.007.

697 Perl, D.P., Gajdusek, D.C., Garruto, R.M., Yanagihara, R.T., Gibbs, C.J., 1982. Intraneuronal aluminum
698 accumulation in amyotrophic lateral sclerosis and Parkinsonism-dementia of Guam. Science,
699 10;217(4564):1053-5. DOI: 10.1126/science.7112111. PMID: 7112111.

700 Pilehvar, A., Town, R.M., Blust, R., 2020. The effect of copper on behaviour, memory, and associative learning
701 ability of zebrafish (Danio rerio). Ecotoxicol. Environ. Saf.;, 188-109900. DOI:
702 10.1016/j.ecoenv.2019.109900.

703 Poléo, A.B.S., Schjolden, J., Sørensen, J., Nilsson, G.E., 2017. The high tolerance to aluminium in crucian
704 carp (Carassius carassius) is associated with its ability to avoid hypoxia. PLoS One; 12(6):e0179519. DOI:
705 10.1371/journal.pone.0179519.

706 Popgeorgiev, N., Bonneau, B., Prudent, J., Gillet, G., 2018. Control of programmed cell death during zebrafish
707 embryonic development. InTechOpen https://doi.org/10.17863/CAM.25035.

708 Quintana-Villamandos, B., González, M.C., Delgado-Martos, M. J., Condezo-Hoyos, L., Böger, R.H.,
709 Lüneburg, N., Pazó-Sayós, L., Gutiérrez-Arzapalo, P.Y., Delgado-Baeza, E., 2016. Short-term Esmolol
710 Attenuates Remodeling of the Thoracic Aorta in Hypertensive Rats by Decreasing Concentrations of ADMA
711 Down-Regulated by Oxidative Stress Eur. J. Pharmacol.; 791, 502-509. DOI: 10.1016/j.ejphar.2016.09.020.

712 Rhee, J.S., Raisuddin, S., Hwang, D.S., Lee, K.W., Kim, I.C., Lee, J.S., 2009. Differential expression of
713 metallothionein (MT) gene by trace metals and endocrine-disrupting chemicals in the hermaphroditic
714 mangrove killifish, Kryptolebias marmoratus. Ecotoxicol. Environ. Saf.; 72(1):206-212. DOI:
715 10.1016/j.ecoenv.2008.06.001.

716 Rodriguez-Fuentes, G., Rubio-Escalante, F.J., Norena-Barroso, E., Escalante-Herrera, K.S., Schlenk, D., 2015.
717 Impacts of oxidative stress on acetylcholinesterase transcription, and activity in embryos of zebrafish (Danio
718 rerio) following Chlorpyrifos exposure. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 172-173, 19-25.
719 DOI: 10.1016/j.cbpc.2015.04.003.

720 Senger, M.R., Seibt, K.J., Cordenonzi, G., Dutra Dias, R., Bogo, M.R., Bonan, C.D., 2011. Aluminum
721 exposure alters behavioral parameters and increases acetylcholinesterase activity in zebrafish (Danio rerio)
722 brain. Cell. Biol. Toxicol.; 27 (3), 199e205. https://doi.org/10.1007/s10565-011-9181-y.

723 Seok, S.H., Baek, M.W., Lee, H.Y., Kim, D.J., Na, Y.R., Noh, K.J., Park, S.H., Lee, H.K., Lee, B.H., Ryu,
724 D.Y., Park, J.H. 2007. Arsenite-induced apoptosis is prevented by antioxidants in zebrafish liver cell line.
725 Toxicol. In Vitro.; 21(5):870-7. DOI: 10.1016/j.tiv.2007.02.011.

726 Shi, X., Du, Y., Lam, P.K., Wu, R.S., Zhou, B., 2008. Developmental toxicity and alteration of gene expression
727 in zebrafish embryos exposed to PFOS. Toxicol. Appl. Pharmacol.; 230(1):23-32. DOI:
728 10.1016/j.taap.2008.01.043.

729 Singla, N., Dhawan, D.K., 2014. Zinc modulates aluminium-induced oxidative stress and cellular injury in rat
730 brain. Metallomics; 6(10),1941–1950. DOI:10.1039/c4mt00097h.

731 Sosa, L.J., Cáceres, A., Dupraz, S., Oksdath, M., Quiroga, S., Lorenzo, A., 2017. The physiological role of the
732 amyloid precursor protein as an adhesion molecule in the developing nervous system. J. Neurochem.;
733 143(1):11-29. DOI: 10.1111/jnc.14122.

734 Stewart, A.M., Braubach, O., Spitsbergen, J., Gerlai, R., Kalueff, A.V., 2014. Zebrafish models for
735 translational neuroscience research: from tank to bedside. Trends Neurosci.; 37:264–278. DOI:
736 10.1016/j.tins.2014.02.011.

737 Strungaru, S., Radojković, P., Dumitru, G., Nicoara, M., Plavan, G., Todirascu-Ciornea, E., 2019. Oxidative
738 stress and changes in swimming performances at zebrafish model (Danio Rerio H. 1822) produced by acute
739 exposure to deltamethrin. Survey in Fisheries Sciences.; 5 (2):121-137. DOI: 10.18331/SFS2019.5.2.12.

740 Tseng, Y.C., Chen, R.D., Lucassen, M., Schmidt, M.M, Dringen, R., Abele, D., PuHwang, P., 2011. Exploring
741 uncoupling proteins and antioxidant mechanisms under acute cold exposure in brains of fish. PLoS One,
742 6(3):e18180. DOI:10.1371/journal.pone.0018180.

743 Vyas, S., Biguet, N.F., Michel, P.P., Monaco, L., Foulkes, N.S., Evan, G.I., Sassone-Corsi, P., Agid, Y., 2002.
744 Molecular mechanisms of neuronal cell death: implications for nuclear factors responding to cAMP and
745 phorbol esters. Mol. Cell. Neurosci.;21(1):1-14. DOI: 10.1006/mcne.2002.1170.

746 Wallin, B., Rosengren, B., Shertzer, H.G., Camejo, G., 1993. Lipoprotein oxidation and measurement of
747 thiobarbituric acid reacting substances formation in a single microtiter plate: its use for evaluation of
748 antioxidants. Anal. Biochem.; 208, 10-5. DOI: 10.1006/abio.1993.1002.

749 Walton, J.R, .Wang, M.X., 2009. APP expression, distribution and accumulation are altered by aluminum in
750 a rodent model for Alzheimer's disease. J. Inorg. Biochem.; 103(11):1548-54. DOI:
751 10.1016/j.jinorgbio.2009.07.027.

752 Wang, W.C., Mao, H., Ma, D.D., Yang, W. X., 2014. Characteristics, functions, and applications of
753 metallothionein in aquatic vertebrates. Front. Mar. Sci.: 1:34. https://doi.org/10.3389/fmars.2014.00034.

754 Westerfield, M., 2000. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), fourth
755 ed. University of Oregon Press, Eugene.

756 WHO Guidelines for Drinking-water Quality; Aluminium in drinking-water. WHO/HSE/WSH/10.01/13.

757 WHO, Geneva, Switzerland, 1997. Environmental health criteria for aluminium. Environmental Health
758 Criteria No.194 pp.xx + 282 pp. ref.many. ISSN: 0250-863X.

759 Xu, J., Zhang, Q., Li, X., Zhan, S., Wang, L., Chen, D., 2017. The effects of copper oxide nanoparticles on
760 dorsoventral patterning, convergent extension, and neural and cardiac development of zebrafish. Aquat.
761 Toxicol.; 188:130-137. DOI: 10.1016/j.aquatox.2017.05.002.

762 Yang, L., Ho, N.Y., Alshut, R., Legradi, J., Weiss, C., Reischl, M., Mikut, R., Liebel, U., Müller, F., Strähle,
763 U., 2009. Zebrafish embryos as models for embryotoxic and teratological effects of chemicals. Reprod
764 Toxicol.; 28(2):245-53. DOI: 10.1016/j.reprotox.2009.04.013.

765 Yokel, R.A., 2020. Aluminum reproductive toxicity: a summary and interpretation of scientific reports. Crit.
766 Rev. Toxicol.; 50(7):551-593. DOI: 10.1080/10408444.2020.1801575.

767 Yousef, M.I., 2004. Aluminium-induced changes in hemato-biochemical parameters, lipid peroxidation and
768 enzyme activities of male rabbits: protective role of ascorbic acid. Toxicology; 199(1),47–57.
769 DOI:10.1016/j.tox.2004.02.014.

770 Zhang, Q.F., Li, Y.W., Liu, Z.H., Chen, Q.L., 2016. Exposure to mercuric chloride induces developmental
771 damage, oxidative stress and immunotoxicity in zebrafish embryos-larvae. Aquat. Toxicol.; 181:76-85. DOI:
772 10.1016/j.aquatox.2016.10.029.

773 Zhang, Y.W., Thompson, R., Zhang, H., Xu, H., 2011. APP processing in Alzheimer's disease. Mol Brain.;
774 4:3. DOI: 10.1186/1756-6606-4-3.

775 Zhao, G., Sun, H., Zhang, T., Liu, J.X., 2020. Copper induce zebrafish retinal developmental defects via
776 triggering stresses and apoptosis. Cell Commun. Signal.; 18(1):45. DOI: 10.1186/s12964-020-00548-3.

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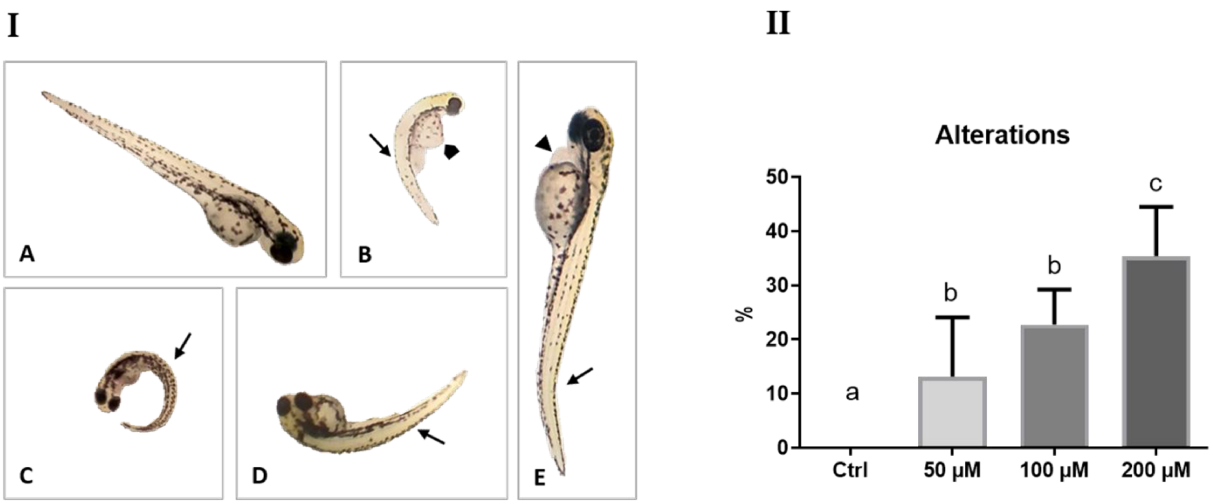
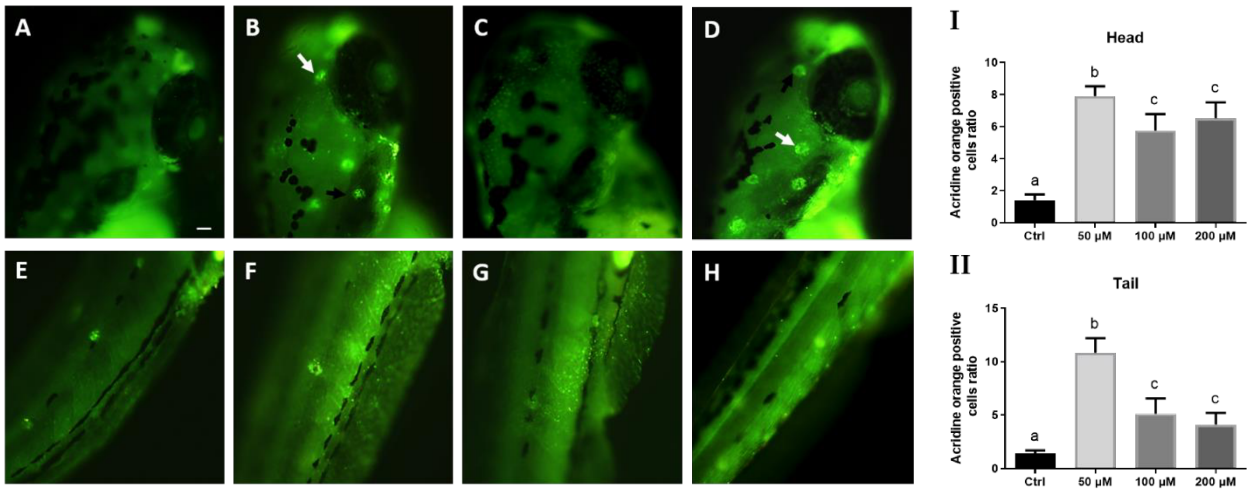


Fig. 1, Panel I: Phenotypical alterations found in zebrafish larvae exposed to 50, 100 and 200 µM of $AlCl_3$ for 72 h. (A) Photomicrograph of control larvae. (B) larva with yolk sac swelling (arrow ▶) (B, C, D, E) larvae with head-tail axis alterations (arrows →). (E) larvae with pericardial edema (arrow ▲). **Panel II**: Percentage of altered larvae exposed for 72 h to 50, 100 and 200 µM of $AlCl_3$. Ctrl: control larvae. Data are expressed as mean±SD. Different lowercase letters indicate significant differences between groups ($p < 0.05$).



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800 **Fig. 2** Whole mount acridine orange staining in zebrafish larvae. (A, E) Head and tail of control
801 larvae. (B, F) Head and tail of larvae exposed to 50 µM $AlCl_3$. (C, G) Head and tail of larvae exposed
802 to 100 µM $AlCl_3$. (D, H) Head and tail of larvae exposed to 200 µM $AlCl_3$. Particular fluorescent
803 cellular accumulations are indicated by arrows. Scale bar: 50 µm. (Graph **I, II**) Acridine orange
804 positive cells ratio of the head (I) and tail (II) of larvae exposed to 50, 100 and 200 µM of $AlCl_3$ for
805 72 h compared to Control (Ctrl). Data are expressed as mean±SD. Different lowercase letters indicate
806 significant differences between groups ($p < 0.05$).

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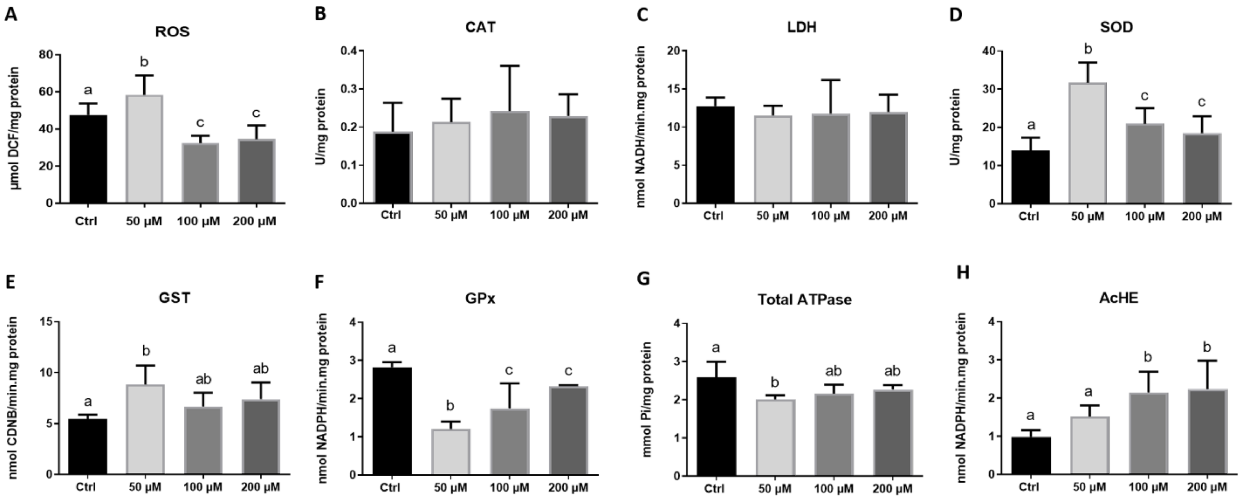

Fig. 3 (A) Reactive oxygen species accumulation (ROS), (B) catalase (CAT) activity, (C) lactate dehydrogenase (LDH), (D) superoxide dismutase (SOD), (E) glutathione S-transferases (GST), (F) glutathione peroxidase (GPx), (G) total ATPase activity, (H) acetylcholinesterase (AChE) activity, in zebrafish larvae exposed to 50, 100 and 200 µM $AlCl_3$ and compared to control (Ctrl). Data are expressed as mean±SD. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

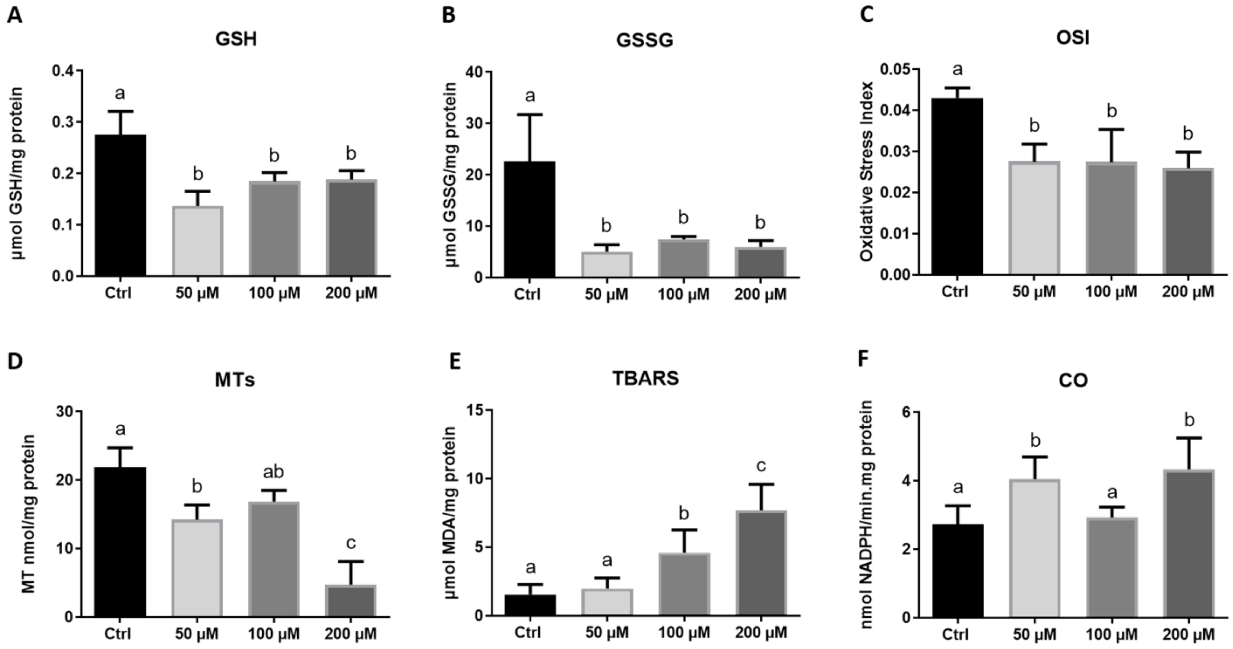

Fig. 4 (A) Levels of reduced glutathione (GSH), (B) oxidized glutathione levels (GSSG), (C) oxidative stress index (OSI), (D) metallothioneins (MTs), (E) lipid peroxidation (TBARS) and (F) oxidative damage to proteins (carbonyls, CO) in in zebrafish larvae exposed to 50, 100 and 200 µM $AlCl_3$ and compared to control (Ctrl). Data are expressed as mean±SD. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

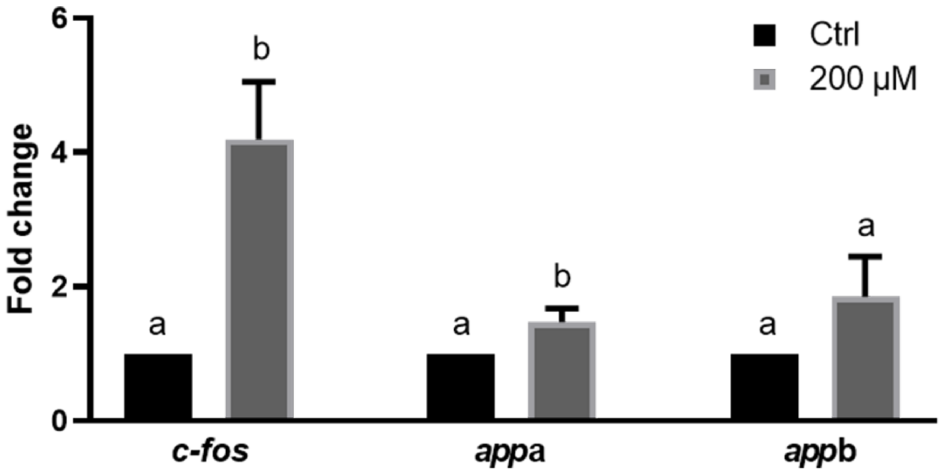

Fig. *5* Expression pattern comparison by means of RT-qPCR**.** Expression level change after 200 µM $AlCl_3$ stimulation for *c-fo*s, *app*a and *app*b. Gene expression level was normalized to the reference transcript *rplp0* and calculated by the $2^{-\Delta\Delta Ct}$ method. Data are expressed as mean of fold change ±S*D*. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

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Exposure to aluminium causes behavioural alterations and oxidative stress in the brain of adult zebrafish

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ABSTRACT

Aluminium (Al) water pollution is an increasing environmental problem. Accordingly, this study aimed to find out more about its toxic effects on aquatic organisms. Adult zebrafish were exposed to 11 mg/L of Al and the behavioural responses and its correlation with brain oxidative stress, antioxidant-defences, changes in metabolism and neurotransmission were assessed at 10, 15 and 20 days of exposure. The behavioural and locomotory responses, suggest an increase in the anxiety state, especially observed in animals exposed to Al for 15 days. The reactive oxygen species increased in a time-dependent trend, while the oxidative damage varied over exposure time. The activity of antioxidant enzymes, as superoxide dismutase, glutathione peroxidase and glutathione S-transferases, and the metallothioneins levels increased after short-term exposures and tended to decrease or stabilize at longer times. The results contribute to understand the toxic mechanisms activated by Al highlighting correlations like behavioural disorders and oxidative state.

1. Introduction

Aluminium (Al) is among the most abundant metals in nature, posing a serious threat to the environment (Tchounwou et al., 2012). It is classified as a dangerous pollutant and can induce toxic effects in terrestrial and aquatic organisms (Alstad et al., 2005; Coleman et al., 2010). However, unlike other metals such as cadmium (Cd) (Favorito et al., 2010, 2017; Ferrandino and Grimaldi, 2008; Ferrandino et al., 2009) or copper (Carotenuto et al., 2020), the Al mechanism of action is less studied, especially in aquatic environment. According to the World Health Organization (WHO), the concentration of Al can vary significantly in natural waters, depending on various physicochemical factors, as mineralogical and pH variations. Normally, dissolved Al concentrations in water range from 0.001 to 0.05 mg/L when the pH is near-neutral values (WHO, 1997, 2021). However, some studies showed that its concentration can vary from 0.014 mg/L in groundwaters to 2.57 mg/L in surface waters (Agarwal et al., 2016) and even rise to 50 mg/L due to acid rains (Agency for Toxic Substances and Disease (ATSDR, 2008) that, changing the acidity of the water, facilitate the solubilisation of the inorganic Al (Lawrence et al., 2007; Zhang et al., 2016). In the last years, the water pollution with Al has been an increasing environmental problem, and a comprehensive analysis of the toxic responses of aquatic organisms is necessary. In this study *Danio rerio,* commonly called zebrafish, was chosen as an animal model organism. Zebrafish is suitable for screening of ecotoxicological effects (Favorito et al., 2011; Santos et al., 2017, 2018) induced by environmental pollutants (Lanzarin et al., 2019; Monaco et al., 2016, 2017a, Motta et al., 2019; Santos et al., 2014). Furthermore, zebrafish is considered an excellent model for motility and neurobehavioral studies (Orger and de Polavieja, 2017) as it has a well-developed central nervous system with numerous characteristics superimposable on the human nervous system (Newman et al., 2011; Sager et al., 2010). In previous studies, we have shown both teratogenic (Monaco et al., 2017b) and behavioural (Capriello et al., 2019) effects induced by waterborne Al on zebrafish larvae. Al is known to affect the oxidative status (Abdel Moneim, 2012; Maheswari et al., 2014; Mesole et al., 2020), in particular, of specific areas of the brain (Sánchez-Iglesias et al., 2009) which, in turn, mediate behavioural phenotypes (de Carvalho et al., 2019). For this reason, this study aimed

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to analyse the effects of Al exposure on behaviour and locomotor activity of adult zebrafish, also paying particular attention to aspects related to oxidative stress induction in the brain, because this is suggested to be a mechanism for the aetiology of motility dysfunction (Tönnies and Trushina, 2017). In this context, the effects of 11 mg/L Al were assessed in adult zebrafish both on locomotor activity and social behaviour after 10, 15 and 20 days of exposure. Moreover, the involvement of reactive oxygen species (ROS) and oxidative damage as a potential mechanism of Al induced toxicity were subsequently analysed by different markers of metal stress. The concentration of 11 mg/L Al was chosen to simulate an environment in which the concentration of aluminium was higher than that normally present in waters (WHO, 1997, 2021). It is known that its concentration can increase due to acid rain that induce Al downwash from the land, increasing its concentration in water resources (Agarwal et al., 2016). However, the Al concentrations found in polluted waters are highly variable (Agarwal et al., 2016; Agency for Toxic Substances and Disease (ATSDR, 2008; Forsido et al., 2020) and few studies are present in the literature on the effects of Al at high concentrations (Awad et al., 2019). For these reasons, this condition seemed the most suitable for our objectives, allowing to simulate what can happen in an aquatic environment polluted by an excessive presence of Al. Nevertheless, to the choice of exposure conditions, the results obtained previously from the survival analysis carried out on zebrafish larvae after Al exposure (Monaco et al., 2017b) were taken into account in order to avoid a high mortality in the treated organisms.

The results contribute to elucidate the effects of aluminium on zebrafish brain and its consequent action on behavioural responses, highlighting also the unknown correlations between behavioural disorders and oxidative state.

2. Material and methods

2.1. Zebrafish housing

Adult zebrafish were housed in rectangular glass tanks with a natural photoperiod of 12:12 h light/dark, at a temperature of 28.5 °C, pH of 7.6 and fed twice a day with a commercial diet (Westerfield, 2000). Fish were maintained at the Department of Biology of the University of Naples "Federico II", according to the guidelines and policies dictated by European regulations on animal of welfare for experimental purposes (Directive 2010/63/EU). The used protocols were approved by the Italian Ministry of Health (Number: 147/2019-PR). All procedures were performed under ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma Aldrich, Germany) to minimize suffering.

2.2. Chemicals

The treatment solution of 11 mg/L of Al was prepared by dissolving directly 2.5 g of $AlCl_3{\cdot}6H_2O$ (Carlo Erba, Italy; CAS no. 7784-13-6) in 25 L of tank water (1/3 bidistilled water, 2/3 tap water).

Unless stated, all other chemicals were of analytical grade and purchased from Sigma (Sigma-Aldrich, Germany).

2.3. Al exposure

Twenty-one fish were divided into three groups of 7 fish. A selection of 7 healthy zebrafish, 4 males and 3 females, of about 6 months were placed in a glass container (43 cm [length] × 24 cm [width] × 36 cm [height]) with 25 L of Al solution (11 mg/L), and exposed respectively for 10, 15 and 20 days. Another group of 7 fish (4 male and 3 female) exposed only to tank water was used as a control. Each treatment was done in triplicate. The temperature of 28.5 °C and pH of 7.6 was kept constant for all tanks. The total exposure solution was changed every day and food debris were removed once a day. At the end of each experimental period, five fish (3 male and 2 female) were random selected for behaviour analysis and locomotor activity. After this analysis, which did not induce any mortality, all fish were sacrificed by an overdose of MS-222 solution (300 mg/L). For biochemical biomarkers analysis, four brains (2 male and 2 female) from independent replicates were used for each treatment group and the control. Other three brains (2 male and 1 female) from each group were carried out for metallothioneins analysis.

2.4. Locomotor activity

To assess changes in locomotor activity, five individual fish (3 male and 2 female) were selected from each group after 10, 15 and 20 days of Al exposure and were analysed, individually, in plastic rectangular tanks (28 cm [length] × 20 cm [width] × 25 cm [height]) containing 8 L of tank water. Data were collected between 10 a.m. and 2 p.m. and after the acclimation period (5 min) and the locomotor behaviour was recorded with a video camera attached to a tripod for 5 min. Subsequently, the videos were analysed using the freely available ToxTrac (Franco-Restrepo et al., 2019; Rodriguez et al., 2018): software Organism video Tracking Application (Umeå University, Linneaus Väg, SE-901 87 Umeå, Sweden Ver.2.84) and compared to the controls. Each video recording was examined for the following mobility characteristics: average speed (m/s), average acceleration (m/s^2), explorative rate (%) and total distance moved (m). The detailed experimental procedures are described in the Supplementary Material.

2.5. Behavioural analysis

The changes in behaviour were assessed by four tests: Light/Dark choice assay, social preference test, shoaling behaviour and mirror test. For each test, five individual fish (3 male and 2 female) were selected from each group after 10, 15 and 20 days of Al exposure and compared their performances with that of the control. In this case, the video-recording was analysed manually by operator because video-tracking may not be able to quantify complex motor patterns and some of these behavioural patterns have been shown to not correlate with swim path characteristics (Blaser and Gerlai, 2006). The Light/Dark test (L/D choice assay) was used to characterize the sensitivity of the adult choice behaviour to lighting conditions and the anxiety of fish after treatment following the methodology described by Lau et al. (2011). Individual animals were introduced into the L/D chamber by netting and allowed to acclimate for 5 min, followed by a 5 min video recording. The rectangular tank (28 cm [length] × 20 cm [width] × 25 cm [height]) containing 8 L of tank water was decorated half with black tape and half with white tape (covering both the bottom and the side walls). The time spent in the light zone and in the dark zone by the organism was evaluated. The social preference test was used to evaluate the changes of zebrafish social preference. Normally, zebrafish is a highly social species. In nature, and in the aquarium, the group of individuals swim close to each other, in group. This group preference, also termed as group cohesion (Engeszer et al., 2007) is useful to study zebrafish sociability by observing the interactions with other fish (Pham et al., 2012; Riehl et al., 2011). For this test we used a common modality: an experimental tank (rectangular, 28 × 20 × 25 cm) where it was allowed the unobstructed view of 2 adjacent stimulus tanks of equal shape and size. On the right side, there was a tank with a group of zebrafish from the same original population of experimental fish, called conspecific tank. An empty tank with tank water was placed on the left side. The experimental fish were placed in the middle tank. Behaviour was recorded for 5 min, after 5 min of adaptation. After recording, the video was analysed and the percentage of time spent near the conspecific tank in the total observation time was evaluated.

The shoaling test was used to evaluate the anxiety of the whole group, following the methodology of Pham et al. (2012). In this case, the test was carried out on all experimental group (7 fish) at the same time. Each group for the several exposure time was placed in a new rectangular 15 L tank containing tank water. The height of the tank was

measured with a ruler, and a red coloured line was drawn, dividing the tank in two equal parts, the deeper and the superficial. After that, a 5 min movie was recorded, after 5 min of adaptation. In this test, the number of individuals present above the drawn line was counted, compared to those present on the lower plane, then expressed as a percentage. An increase in time spent near the bottom reflects the presence of an anxiety state. The mirror test was used to evaluate the aggressiveness of fish, because solitary fish when encounter another fish often exhibit agonistic behaviours. In this case, the "approaching" mirror image was expected to elicit aggression (Gerlai et al., 2008). For this test, a mirror was placed in one side of the test tank (28 × 20 × 25 cm) containing 8 L of tank water. Subsequently, an individual fish was added for acclimation. Recording started 5 min later and behaviour was monitored and recorded for five min. Following recording, the video was analysed, determining the percentage of time spent by fish investing against the mirror on the total observation time.

2.6. Sample collection and protein quantification

For biochemical analysis, samples (4 brains) from the replicates of each experimental group were collected separately, homogenized and prepared for analysis as previously described by Félix et al. (2018). For metallothioneins (MTs) assay, other 3 brains groups from the replicates of each experimental group, were collected separately and properly processed for analysis. Protein quantification, necessary to normalize assayed enzymatic activities, was performed using the BioTek's Take3™ Micro-Volume Plate by optical absorbance at 280 nm using bovine serum albumin (BSA) as standard. All detailed experimental procedures are described in the Supplementary Material.

2.7. ROS accumulation and determination of ATPase activity

ROS accumulation was estimated by a previous methodology (Deng et al., 2009; Félix et al., 2018). ATPase activity was measured as explained by Ames (1966) and Lança et al. (2015). For the detailed experimental procedures of both parameters see the Supplementary Material.

2.8. Enzyme activities

All enzymatic assays were carried out with PowerWave XS2 microplate scanning spectrophotometer (Bio-Tek Instruments, USA) at 30 °C. All samples were measured against a reagent blank and performed in duplicate as referred in supplementary files. To evaluate the superoxide dismutase (SOD) activity, it was followed the protocol of Durak et al. (1993). Catalase (CAT) activity was determined by protocol by Claiborne (1985). Glutathione peroxidase (GPx) activity was determined spectrophotometrically at 340 nm by the method described by Massarsky et al. (2017). The glutathione S-transferases (GST) was assayed as described by Habig and Jakoby (1981). Lactate dehydrogenase (LDH) is a key enzyme in anaerobic metabolism, and its activity was tested by the method described by Domingues et al. (2010). Acetylcholinesterase (AChE) is a key enzyme in neurotransmission and it was analysed as described by Rodriguez-Fuentes et al. (2015). All detailed experimental procedures are described in the Supplementary Material.

2.9. Glutathione levels and oxidative stress index

The reduced (GSH) and oxidized (GSSG) glutathione levels were evaluated fluorometrically, at 320 nm and 420 nm, respectively excitation and emission wavelengths (Misra and Niyogi, 2009), with the fluorochrome ortho-phthalaldehyde (OPT) (1 mg/mL methanol), in a Varian Cary Eclipse (Varian, USA) spectrofluorometer equipped with a microplate reader. See Supplementary Material for detailed protocol. The oxidative-stress index (OSI) was calculated according to the quotient between GSH (μmol of GSH/mg protein) and GSSG (μmol of GSH/mg protein).

2.10. Oxidative damage biomarkers and metallothioneins assay

The lipid peroxidation (TBARS) was determined by the thiobarbituric (TBA) method described by Wallin et al. (1993). The oxidative damage to proteins (carbonyls, CO) was evaluated by the DNPH (2, 4-dinitrophenylhydrazine) method described by Quintana-Villamandos et al. (2016). The metallothioneins assay was carried out according to the indications described by Linde and Garcia-Vazquez (2006). Detailed protocols were reported in Supplementary Material.

2.11. Statistical analysis

A sample size calculation was performed with the G*Power 3 (University of Düsseldorf, Germany) based on standard deviations (Oliveira et al., 2013). Before hypothesis testing, the normal distribution and homogeneity of the data were confirmed by Shapiro-Wilk and Levene's tests, respectively. Statistically significant differences among treatments were assessed by one-way analysis of variance ANOVA followed by Tukey's pairwise comparison tests with the aid of GraphPad Prism 7 (GraphPad Software, La Jolla California USA, www.graphpad.com). Data were expressed as mean ± SD and were considered to be statistically significant $p < 0.05$.

3. Results

3.1. Locomotor activity

Al induced mostly similar effects on all parameters of locomotion tested for all specimens regardless of sex. The velocity (Fig. 1A) was 0.33 ± 0.07 m/s in the control group and remained constant in organisms exposed to Al (0.31 ± 0.04 m/s) during 10 days. It increased significantly, after 15 days (0.45 ± 0.05 m/s; $p < 0.05$), decreasing thereafter, at 20 days of treatment (0.40 ± 0.05 m/s). Although the acceleration (Fig. 1B) showed a similar trend, no significant differences were observed between animals exposed during 10 days (0.60 ± 0.20 m/s^2) and those of control group (0.82 ± 0.17 m/s^2). A significant increase in acceleration was reported in organisms exposed to Al for 15 days (1.04 ± 0.32 m/s^2; $p < 0.05$) and a decrease after 20 days (0.92 ± 0.21 m/s^2). Also, the distance moved (Fig. 1C) had a trend similar to that of the other evaluated parameters. Control animals travelled on average 45.37 ± 6.44 m, distance not significantly different from that travelled by fish exposed during 10 days (36.52 ± 6.87 m). This distance was significantly higher in organisms exposed during 15 days (54.49 ± 13.04 m; $p < 0.01$), partially returning to control levels after 20 days (50.94 ± 7.67 m). The exploration rate was evaluated taking into consideration the explored area and time (Fig. 1D). It had a trend superimposable to the previous ones. The controls showed an exploration rate of 40.88 ± 6.0 %, that not changed significantly after 10 days (33.52 ± 6.09 %) of exposure to Al but increased significantly in fish treated for 15 days (49.22 ± 8.31 %; $p < 0.01$). After 20 days, the exploration rate decreased (40.05 ± 4.95 %) to values similar to that shown by the control group.

3.2. Behavioural testing

In the light/dark test (Fig. 2A), the treatment with Al produced a significant difference in comparison to the behaviour of control fish (81 ± 5.81 %) that remained in the dark part of the tank for almost all the assay time. The most significant differences occurred after 10 and 15 days of treatment, with a reduction in the time spent in the dark part in favour of the light, on average 43.65 ± 21.30 %; $p < 0.001$ of and 56 ± 13.11 %; $p < 0.05$ respectively. After 20 days of treatment, no significant differences from of the control group were perceived, with a mean time spent in the dark part of 62.33 ± 15.82 %. In the social preference test

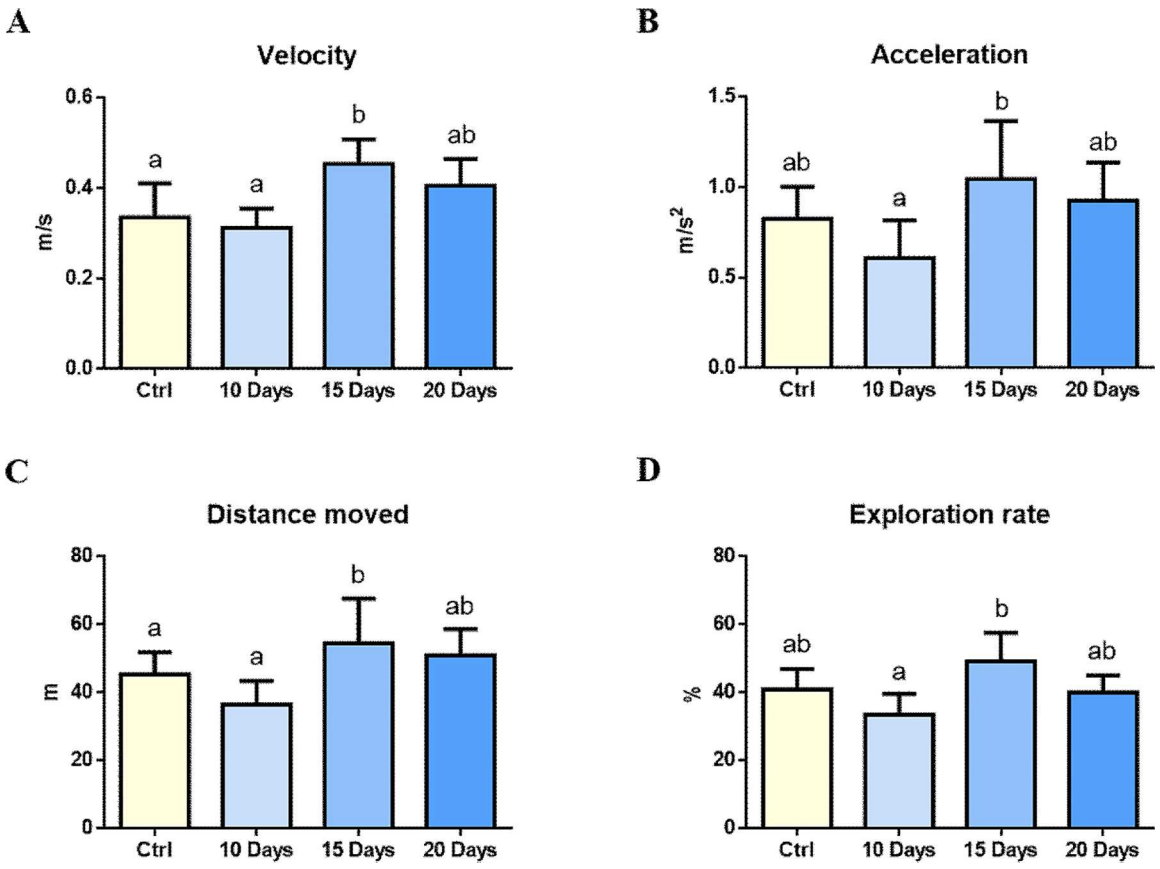

Fig. 1. Locomotor activity of adult zebrafish from control group and exposed to Al during 10, 15 and 20 days, analysed by ToxTrac software. (A) Velocity test (m/s). (B) Acceleration test (m/s^2). (C) Distance moved (m). (D) Exploration rate (%). Data are expressed as mean ± SD. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

(Fig. 2B), it was clear that the control fish spent almost all of the time (90 ± 4.54 %) trying to reach the conspecific tank. For groups exposed during 10 days to Al, there was not a significant decrease in the time spent near the conspecific tank, on average 80.86 ± 20.66 %. While a greater difference and significant isolation was recorded for those fish exposed for 15 days to Al, who spent only 54.14 ± 11.60 %; $p < 0.01$ of the time trying to reach their group of origin, while the remaining time was spent near the empty tank. However, this difference was less evident in fish exposed during 20 days to the treatment, who spent on average 68.74 ± 17.18 % near the conspecific tank. In the shoaling test (Fig. 2C), a linear decrease occurred with exposure time, in the number of fish that spent the analysis time on the upper part of the tanks rather than on the bottom. On average, 87.61 ± 7.63 % of the control group spent time in the top of the tank. After 10 days of treatment with Al only 63.33 ± 3.94 %; $p < 0.01$ of the group remained in the upper part, while the other half preferred the lower part of the tank. After 15 days the percentage of fish in the upper part decreased significantly, with an average of 49.66 ± 9.33 %; $p < 0.001$ which remained constant even in the twentieth day of treatment (50.77 ± 6.50 %; $p < 0.001$). These behavioural performances were similar for organisms in the same group regardless of sex of analysed specimen. In the mirror test (Fig. 2D) there was no significant toxicological effect on the aggressiveness of organisms. The organisms exposed to Al during 10 days spent, in mean, 39.5 ± 26.16 % of time near the mirror, the controls about 52.83 ± 24.12 % and the other treatment periods showed a mean respectively of 48.26 ± 12.30 % for fish exposed during 15 days and 44.4 ± 23.61 % when exposed for 20 days.

3.3. ROS levels and ATPase activity

The generation of ROS increased along the time of exposure to Al with a significant increase ($p < 0.05$) of approximately double in organisms exposed to 20 days of treatment compared to controls (Table 1). The ATPase activity was measured from the inorganic phosphate (Pi) liberated from ATP. The Pi released was significantly halved after 10 days of treatment ($p < 0.05$) compared to control fish. The activity seemed almost physiological after 15 and 20 days of treatment, albeit the changes observed (Table 1). All analyses carried out for the evaluation of the oxidation state of zebrafish brain did not show changes related to the sex of the specimens.

3.4. Enzyme activities

The effects of exposure to aluminium during 10, 15 and 20 days on the activities of SOD, GST, GPx, LDH showed a similar trend, with a significant increase in its activity observed in fish exposed during 15 days, when compared to controls and the other times of treatment. The activity of SOD increased about 1.7-fold after 15 days of treatment ($p < 0.05$) than the activity registered in the brain of control fish. Instead, after 10 and 20 days of treatment, the levels were similar to those recorded in the control group. Similarly, the GST levels increased about 1.6-fold after 15 days of treatment ($p < 0.05$) compared to the control group but no significant difference was evidenced after 10 and 20 days of treatment. The GPx activity was even tripled after 15 days ($p < 0.01$) compared to those measured in the controls and the other treated groups. Also, the LDH activity followed the same trend, although the difference between the average activity observed in the brains of the control fish and those exposed to Al during 15 days ($p < 0.05$) was less

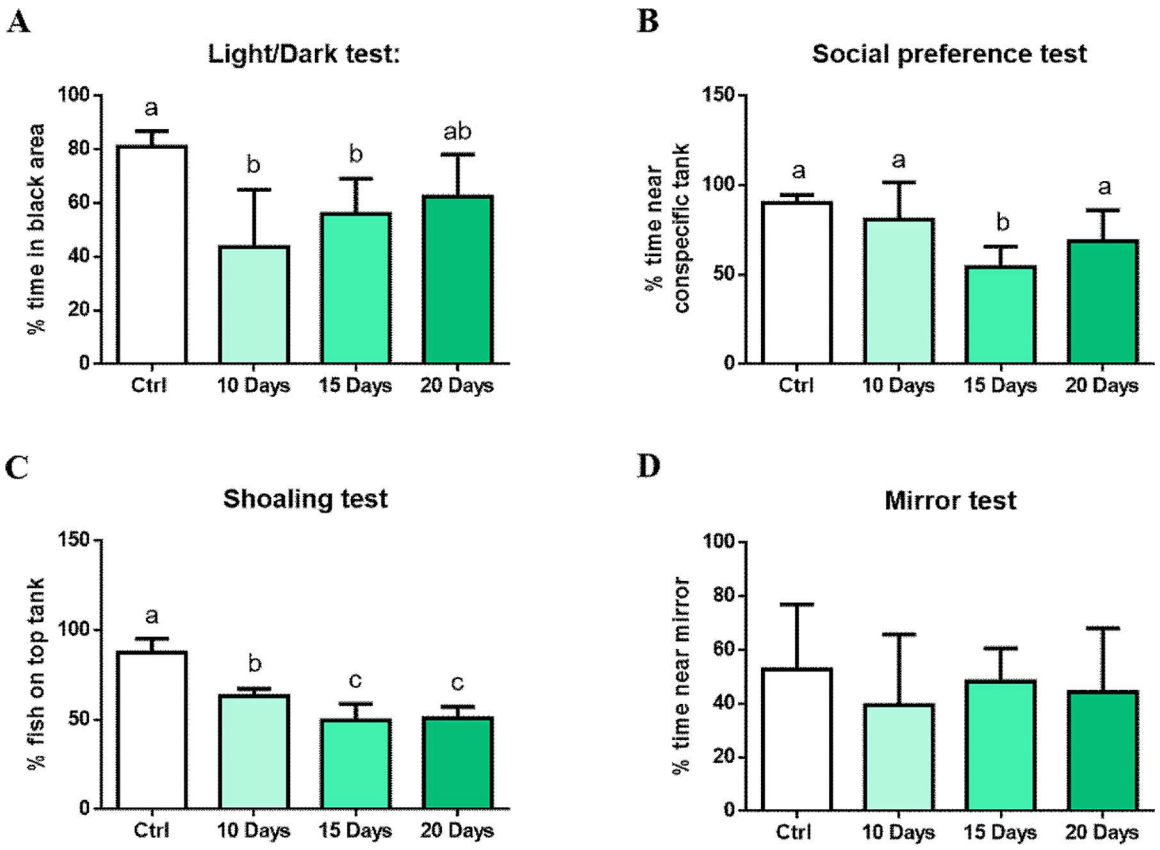

Fig. 2. Behavioural analysis of zebrafish from the control group and exposed to Al during 10, 15 and 20 days. (A) Light/Dark test. (B) Social preference test. (C) Shoaling test. (D) Mirror test. Data are expressed as mean ± SD. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

Table 1
Biochemical parameters evaluated in zebrafish brain of fish exposed to aluminium during 10, 15 and 20 days and compared to control (Ctrl). Data are expressed as mean ± SD. Different lowercase letters indicate significant differences between groups.

Biochemical parameters	Ctrl	10 Days of Al	15 days of Al	20 days of Al
ROS (μmol of DCF/mg protein)	51.10 ± 8.84^{a}	58.70 ± 13.88^{a}	73.88 ± 1888^{ab}	90.74 ± 32.77^{b}
Total ATPase (mmol of Pi/mg protein)	4.78 ± 0.77^{a}	2.75 ± 0.59^{b}	4.25 ± 1.34^{a}	3.47 ± 0.50^{ab}
SOD (U/mg protein)	29.37 ± 10.77^{a}	20.58 ± 7.61^{a}	50.78 ± 15.18^{b}	25.65 ± 7.52^{a}
GST (nmol CDNB/min mg protein)	9.93 ± 3.58^{a}	7.18 ± 4.03^{a}	15.95 ± 3.97^{b}	6.81 ± 3.23^{a}
GPx (nmol NADPH/min mg protein)	3.78 ± 1.66^{a}	1.50 ± 0.42^{a}	10.63 ± 2.29^{b}	2.61 ± 0.53^{a}
LDH (nmol NADH/min mg protein)	45.54 ± 13.92^{a}	39.80 ± 15.71^{a}	62.43 ± 17.27^{b}	32.21 ± 4.59^{a}
AChE (nmol NADH /min mg protein)	1.24 ± 0.33^{a}	0.93 ± 0.15^{ab}	0.78 ± 0.34^{ab}	0.46 ± 0.13^{b}
CAT (U/mg protein)	0.23 ± 0.10	0.45 ± 0.23	0.27 ± 0.16	0.37 ± 0.11
GSH (μmol GSH/mg protein)	0.046 ± 0.01^{a}	0.17 ± 0.02^{b}	0.04 ± 0.02^{a}	0.09 ± 0.03^{c}
GSSG (μmol GSSG/mg protein)	3.61 ± 1.48^{a}	7.64 ± 3.38^{b}	3.25 ± 1.31^{a}	4.86 ± 0.64^{ab}
OSI	0.025 ± 0.003^{a}	0.044 ± 0.009^{b}	0.024 ± 0.004^{ab}	0.029 ± 0.013^{ab}
TBARS (μmol MDA/mg protein)	0.107 ± 0.08^{a}	0.81 ± 0.20^{b}	0.37 ± 0.12^{a}	0.61 ± 0.19^{b}
CO (nmol NADPH/min mg protein)	0.403 ± 0.030^{a}	0.450 ± 0.112^{ab}	0.932 ± 0.245^{b}	0.670 ± 0.264^{ab}
MTs (μmol MTs/mg protein)	0.37 ± 0.09^{a}	0.63 ± 0.06^{b}	0.35 ± 0.10^{a}	0.40 ± 0.11^{a}

than in the previous cases. Instead, Acetylcholinesterase (AChE) showed a time-dependent decrease trend, although significance levels were just reached after 20 days ($p < 0.05$) of Al exposure, when compared to control with a difference of 2.7-fold. At 10 and 15 days of treatment, the values were similar to those observed in the control. The CAT activity was shown to be irregular with no significant differences among control and treated groups. All values are presented in Table 1.

3.5. Glutathione levels and oxidative stress index

The reduced glutathione levels (GSH) had a fluctuating trend. The GSH levels increased by about double after the maximum exposure time of 20 days ($p < 0.05$) compared to the controls, but they had even increased by about 4-fold after 10 days of treatment ($p < 0.001$). By contrast, for 15 days exposure period the levels were similar to those recorded in the control brains. Instead, the oxidized glutathione levels (GSSG) increased significantly by double after 10 days of treatment ($p < 0.05$), compared to control levels and unlike its reduced form, oxidized glutathione levels were similar to controls at both 15 and 20 days. Relatively to the oxidative stress index (OSI), the trend followed that already seen for GSSG with a significant difference measured only between controls and those exposed to Al for 10 days ($p < 0.05$). All values are presented in Table 1.

3.6. Oxidative damage biomarkers and metallothioneins assay

The lipid peroxidation (TBARS) increased in all treated groups compared to the control. However, significant differences were observed in the fish exposed for 10 days to Al ($p < 0.01$) for which the level was 7.6 times greater than those controls. For the maximum exposure time (20 days), there was also higher levels of lipid peroxidation ($p < 0.05$), about 6-fold that of the control group. Finally, after 15 days of exposure, there was no significant differences compared to the control group. On the contrary, the oxidative damage of proteins (carbonyls, CO) was particularly high in the brains of fish exposed to treatment during 15 days ($p < 0.05$) compared to control. On the other hand, for those exposed during 10 and 20 days, the levels were not significantly different from those measured in the control group. The level of metallothioneins significantly increased, by 1.7-fold in the brains of fish exposed to Al for 10 days ($p < 0.05$) compared to controls. Instead, the quantity recorded in the other two exposure times showed no differences to the control group. All values are presented in Table 1.

4. Discussion

The goal of this study was to evaluate the effects of Al exposure on adult specimens of zebrafish, by studying locomotor and behaviour activity and the correlation of their performances with the brain oxidative state, after different exposure periods. Having used an inorganic compound ($AlCl_3{\cdot}6H_2O$), it is necessary to specify that the pathogenicity and the alterations found in this study are to be attributed to aluminium, because the chlorine dose released with the used concentration (0.1 g/L $AlCl_3{\cdot}6H_2O$), is harmless for zebrafish as demonstrated by Kent et al. (2014). The results of this study show a similar trend in outcomes of locomotor activity with increased activities after 15 days of exposure, which remained mostly constant after 20 days. This trend of parameters seems to be connected to increase in stress and anxiety, confirmed by the results of the behavioural tests. Normally, the adult zebrafish manifest anxiety and stress increasing the movements, the speed and other associated parameters (Egan et al., 2009; Kalueff et al., 2013). In the Light/Dark behavioural adaptation test, it is known that zebrafish exhibit a light avoidance behaviour to protect itself from potential predators, taking refuge in the dark (Aponte and Petrunich-Rutherford, 2019; Lau et al., 2011). In this study, the organisms exposed spent more time in the light zone than in the dark ones. This phenomenon is linked to a greater anxiety-inducing state (Blaser and Rosemberg, 2012) that increased in the treated organisms especially for the shorter exposure times. Considering the social preference test, the fish generally prefer to spend more time close to a conspecific tank (where there are other fish) spending over 70–90 % of time there (Pham et al., 2012). Instead, after 15 days of exposure to Al, the organisms showed an increase in the time spent in solitude, consistent with the stress mode induced. Also, the results of the shoaling test showed an increase of the anxious and stressed state. Normally, the fish tend to stay in the top of the tank, searching for food (Engeszer et al., 2007) but after treatment with Al, a decrease in the time spent by zebrafish in the top half of the tank was observed. The results are in agreement with other studies of exposure to anxiogenic factors (Cachat et al., 2010) or other metals (Dipp et al., 2018; Pilehvar et al., 2020). The mirror test is useful to evaluate aggression that manifests as reduced distance of organism from mirror image (Gerlai et al., 2008; Way et al., 2015). In this case, comparing to the control group, there was no increase in agonistic behaviour of zebrafish after Al exposure, probably because, this treatment does not affect the aggressive phenotype of the chosen model organism. The hyperresponsiveness showed may be in line with the hyperactivity of locomotion observed by Ruiter et al. (2016) after Cd exposure but also may be caused by altered neuronal signalling, such as that observed in rats, in which the Cd-induced changes in the neurotransmitters dopamine and 5-hydroxytryptamine (Antonio et al., 1998). Moreover, the decrease or the stabilization observed after a longer period of exposure may be due to activation of an adaptive process. Indeed, an adaptive response is not uncommon in zebrafish after exposure to metals as demonstrated by Jijie et al. (2020) and by Bui Thi et al. (2020). Another possible explanation for this effect is the time required for regulatory and detoxification mechanisms to become active (Pilehvar et al., 2020). The present study provides evidence that Al inhibits AChE activity, an enzyme that catalyses the breakdown of ACh (Soreq and Seidman, 2001), acting as a cholinotoxin, as demonstrated by Senger et al., 2011. However, some authors report decreases in AChE activity (Kumar, 1999) whereas others report an activation of AChE in the presence of Al (Peng et al., 1992; Zatta et al., 2002). In this study, the AChE decrease could be associated with the increase of neurotransmitter ACh. ACh, in fact, produces an excitatory effect on neuromuscular activity and can cause alterations in motor parameters (Mandl and Kiss, 2007). Despite the various evidences collected the mechanism of action of Al is still little-known today but it is possible to hypothesize that impaired of swimming and behavioural performance are linked to the alteration of the oxidant-antioxidant equilibrium. Mitochondria are the major site for ROS generation and the key intracellular targets for stress inducers (Bhansali et al., 2017). In this study, the significant presence of ROS observed during the treatment can be attributed to the enhanced mitochondrial activity and the increased electron chain activity (Abdel Moneim, 2012). The continue Al exposure could induce oxidative stress through the indirect production of ROS via Fenton reaction (Exley, 2004) and by the stabilization of superoxide ion (Mujika et al., 2014; Ruiter et al., 2016). This evidence could be connected to the decrease of antioxidant enzymes at the 20^{th} day of treatment although the quantity of ROS remained stably high. However, no evidence is currently known to support this theory in a biological system. Mitochondria also provide the cell with adenosine triphosphate (ATP) (Wang et al., 2016). It is known that Cd induces mitochondrial swelling and decreases mitochondrial ATP synthesis (Pan et al., 2018). It can be that this also occurred after a short exposure to Al. The ATPase is involved in ROS signalling (Srikanthan et al., 2016), which could explain the improvement of its activity after 15 days of treatment in conjunction with the increase in the activity of antioxidant enzymes. Furthermore, its activity is also necessary to support the increase in locomotor activity, which was observed after 15 days of exposure to Al. LDH is an important enzyme in neuronal physiology (Zenki et al., 2014) and it is widely used as a marker of organ or tissue damage (Oliveira et al., 2016; Silva Santos et al., 2018). In this study, the level of LDH increased after 15 days of treatment following the trend of the motility parameters. This suggests that pyruvate, the final product of glycolysis, was preferentially used to produce lactate to cope with the high and rapid demand of energy (Frasco and Guilhermino, 2002). Although it is less efficient, anaerobic glycolysis is 100 times faster than oxidative phosphorylation enabling it to fulfil the short-term energy requirements (Valvona et al., 2016). In this case, at the same time of exposure (15 days), both LDH and ATPase levels were increased. It is known that some types of cells, use LDH to elevate the rate of glycolysis, ATP and lactate production, a reaction known as Warburg effect (Kim and Dang, 2006). However, no evidence of this is known following metal-induced oxidative stress. Furthermore, the LDH levels decreased significantly after 20 days of treatment compared to the previous sampling time-point (15 days); while the ATPase levels did not change between the two exposure times. Probably after 20 days of treatment, the activation of only one mechanism had been able to satisfy the energy demand, which was stabilizing over time. Considering the antioxidative physiology in fish brains, SOD, CAT, and GPx are considered to be the first line of defence against oxidative stress (Ighodaro and Akinloye, 2018). In this study, there was an evident increase in the activities of SOD and GPx, especially after 15 days of exposure, probably in response to increased free radical production. Subsequently, the decreased activity might have been resulted both to the oxidative modification of genes that control these enzymes and to Al binding at their active site (Ruiter et al., 2016). Indeed, Al, like Cd can compete with the endogenous metals for the active site of antioxidant

enzymes and metalloproteins, inducing the inactivation of defence mechanisms (Ruiter et al., 2016). In this way, their inactivation can contribute to less efficient degradation of endogenous ROS and a consequent accumulation. Furthermore, it is known that the alteration of GPx activity is a reason for brain senescence (Kishido et al., 2007). In this case, the alterations could be related to the recorded behavioural anomalies. In addition, Al exposure induced alterations of the levels of the (interdependent) GSH and GSSG. GSH plays a crucial role in cytoplasmatic ROS regulation by reducing protein disulfides and other cell molecules (Harvey et al., 2002) to maintain redox homeostasis (Félix et al., 2016). The aluminium-dependent increase of GSH after 10 days of treatment was accompanied by an increase of GSSG, leading to an increased ratio of GSH to GSSG. However, their following decrease in the subsequent exposure stages is probably due to an activation of oxidative defences, such as the antioxidant enzymes SOD or GPx. This trend finds broad support in the classification of the induced oxidative stress intensity proposed by Lushchak (2014). Typically, as indicated by Massarsky et al. (2017) and Adeyemi et al. (2015) the concentration of GSH decreases in zebrafish in response to induction of oxidative stress, but not all exposures result in GSH decrease. For example, exposure to antidepressant amitriptyline resulted in a U-shape response (Yang et al., 2014), a phenomenon also observed here, albeit with an opposite trend. Moreover, some discrepancy between the levels of GPx and GSH-GSSG was observed, which have already been noted in zebrafish (Jin et al., 2015) and rat (Jihen el et al., 2010). In fact, the conversion of GSH to GSSG occurs both during GPx-catalysed reduction of H_2O_2 and in spontaneous reactions with free radicals (Li et al., 2000; Meister, 1994). Moreover, as indicated by Massarsky et al. (2017) GSH can act independently of GPx to neutralize ROS. Lastly, GSH is an important substrate of GST, an important detoxifying enzyme within phase II xenobiotic metabolism. Therefore, decreases in GSH could also be attributed to the induction of phase II metabolism to render certain chemicals more water-soluble (Massarsky et al., 2017). This would justify the increase in the concentration of GST only after 15 days of treatment and not before. GST catalyses the conjugation reactions of xenobiotics to GSH, protecting cells from oxidative stress (Larose et al., 2008). Probably, the increased GST activity after 15 days of treatment was a protective response to Al to increase the formation of conjugates between Al^{3+} and GSH as described by Singla and Dhawan (2014). Subsequently, after 20 days of treatment, the activity of GST, like those of other antioxidant enzymes (SOD and GPx), decreased significantly to values similar to the ones observed in the control group. Both GST and GPx use GSH as substrate, which consequently increases significantly at 20^{th} day. An oscillation between the levels of these antioxidants, albeit so related to each other, is not uncommon in zebrafish, as also verified by Jin et al. (2015) after exposure to other metals such as cadmium and chromium.

The MTs are intracellular proteins cysteine-rich, that acts as scavengers (Chen et al., 2014) in the detoxification processes (Chen et al., 2004; Ghoshal and Jacob, 2001). In our study, the exposure to Al during 10 days was accompanied by an increase of MTs levels. Probably, this is due to a cytoprotection mechanism (Coyle et al., 2002). Indeed, chelation of Al by MT attenuate the direct ROS production, enhancing metal ion export out of cells and reducing its uptake (Park et al., 2001). The redox function of MTs is associated to GSH/GSSG because they set the transfer of metal between MTs and metal-binding proteins (Agrawal et al., 2014; Ghorbel et al., 2016). This would justify the similar trend between the parameters. After 20 days of treatment, also the levels of metallothioneins decreased probably because after a long-term exposure, trace metal accumulation might have reached a toxic threshold limit and thus MT biosynthesis was compromised as demonstrated by study of Cho et al. (2006); De Smet et al. (2001) and Van Campenhout et al. (2010). Regarding the toxic effects on cellular components, Al cannot initiate peroxidation but it can attack almost all cell components including membrane lipids (Flora et al., 2003). Lipid peroxidation plays an important role in the toxicity and it represents a manifestation of oxidative damage of many xenobiotics (Yousef, 2004). In the present study, Al exposure induced a significant increase in TBARS, in brain, after 10 days of exposure probably due to free radical-induced oxidative cell injury. Furthermore, this increase may result from the inactivation of some antioxidant enzymes leading to membrane damage (Abdel Moneim, 2012; Nehru and Anand, 2005). This hypothesis is also reflected in the subsequent treatment phases. The decrease in the TBARS levels registered after 15 days coincides with a significant increase in the production of these antioxidants as SOD, GST, GPx. On the other hand, CO levels increased only after 15 days of treatment, decreasing thereafter, at day 20. This phenomenon was also previously observed in zebrafish brain following oxidative stress (Tseng et al., 2011). Moreover, it is possible to note that lipid peroxidation was detected in previous periods than the carboxylation of proteins, probably because carbonyls proteins are more stable than TBARS (Bizzozero, 2009). Indeed, carbonyls are relatively difficult to induce and they are normally detected only in conditions of considerable oxidative stress (Levine, 2002).

5. Conclusion

The study showed a relationship between impairment of zebrafish swimming behaviour and the generation of free radicals, which resulted in the induction of antioxidant activities, whose role in zebrafish is to prevent or reduce the effects of ROS. The significant rise in antioxidant enzymes activities were mainly recorded after 15 days of exposure to waterborne Al, with a reduction of these parameters after 20 days. This is supported also by the behavioural and locomotor analyses variables that at the 15^{th} day were altered and that after 20 days showed a recovery, due probably to a decreased oxidative stress intensity. Social phenotypes are a key part of zebrafish natural behaviour and examining shoaling phenotypes, social preference, and responses to mirror, contribute to better understand social behaviours as well as stress and anxiety in adult zebrafish. For these reasons, this study contributes to elucidating the effects of aluminium on zebrafish brain and its consequent action on behavioural responses.

Author contributions

Teresa Capriello: Conceptualization, Methodology, Formal analysis, Writing - Original Draft;

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Declaration of Competing Interest

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Appendix A. Supplementary data

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References

Abdel Moneim, A.E., 2012. Evaluating the potential role of pomegranate peel in aluminum-induced oxidative stress and histopathological alterations in brain of female rats. Biol. Trace Elem. Res. 150 (1-3), 328–336. https://doi.org/10.1007/s12011-012-9498-2.

Adeyemi, J.A., da Cunha Martins-Junior, A., Barbosa, F.Jr., 2015. Teratogenicity, genotoxicity and oxidative stress in zebrafish embryos (Danio rerio) co-exposed to arsenic and atrazine. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 172-173, 7–12. https://doi.org/10.1016/j.cbpc.2015.04.001.

Agarwal, S., Tyagi, I., Gupta, V.K., Dehghani, M.H., Ghanbari, R., 2016. Investigating the residual aluminum elimination from conventional and enhanced coagulation by phosphate compounds in wastewater treatment process. J. Mol. Liq. 221, 673–684. https://doi.org/10.1016/j.molliq.2016.06.051.

Agency for Toxic Substances and Disease (ATSDR), 2008. Toxicological Profile for Aluminum. CAS, 7429-90-95.

Agrawal, S., Flora, G., Bhatnagar, P., Flora, S.J., 2014. Comparative oxidative stress, metallothionein induction and organ toxicity following chronic exposure to arsenic, lead and mercury in rats. Cell Mol. Biol. (Noisy-le-grand) 60 (2), 13–21.

Alstad, N.E., Kjelsberg, B.M., Vøllestad, L.A., Lydersen, E., Poléo, A.B., 2005. The significance of water ionic strength on aluminium toxicity in brown trout (Salmo trutta L.). Environ. Pollut. 133 (2), 333–342. https://doi.org/10.1016/j.envpol.2004.05.030.

Ames, B.N., 1966. Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol. 8, 115–118. https://doi.org/10.1016/0076-6879(66)08014-5.

Antonio, M.T., Benito, M.J., Leret, M.L., Corpas, I., 1998. Gestational administration of cadmium alters the neurotransmitter levels in newborn rat brains. J. Appl. Toxicol. 18 (2), 83–88. https://doi.org/10.1002/(sici)1099-1263(199803/04)18:2<83::aid-jat480>3.0.co;2-1.

Aponte, A., Petrunich-Rutherford, M.L., 2019. Acute net stress of young adult zebrafish (Danio rerio) is not sufficient to increase anxiety-like behavior and whole-body cortisol. Peer J. 7, e7469. https://doi.org/10.7717/peerj.7469.

Awad, K.M., Salih, A.M., Khalaf, Y., Suhim, A.A., Abass, M.H., 2019. Phytotoxic and genotoxic effect of Aluminum to date palm (Phoenix dactylifera L.) in vitro cultures. J. Genet. Eng. Biotechnol. 17 (1), 7. https://doi.org/10.1186/s43141-019-0007-2, 21.

Bhansali, S., Bhansali, A., Dhawan, V., 2017. Favourable metabolic profile sustains mitophagy and prevents metabolic abnormalities in metabolically healthy obese individuals. Diabetol. Metab. Syndr. 9, 99. https://doi.org/10.1186/s13098-017-0298-x.

Bizzozero, O.A., 2009. Protein carbonylation in neurodegenerative and demyelinating CNS diseases. Handbook of Neurochemistry and Molecular Neurobiology. Springer US, pp. 543–562. https://doi.org/10.1007/978-0-387-30375-8_23.

Blaser, R., Gerlai, R., 2006. Behavioral phenotyping in Zebrafish: comparison of three behavioral quantification methods. Behav. Res. Meth. 38, 456–469. https://doi.org/10.3758/bf03192800.

Blaser, R.E., Rosemberg, D.B., 2012. Measures of anxiety in zebrafish (Danio rerio): dissociation of black/white preference and novel tank test. PLoS One 7, e36931. https://doi.org/10.1371/journal.pone.0036931.

Bui Thi, N.H., Nguyen Thi, N.A., Audira, G., Siregar, P., Liang, S.T., Huang, J.C., Hsiao, C. D., 2020. Chronic exposure to low concentration lead chloride-induced anxiety and loss of aggression and memory in zebrafish. Int. J. Mol. Sci. 21 (5) https://doi.org/10.3390/ijms21051844, 7.

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K.M., Wu, N., Wong, K., Roy, S., Suciu, C., Goodspeed, J., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Tan, J., Denmark, A., Gilder, T., Kyzar, E., Dileo, J., Frank, K., Chang, K., Utterback, E., Hart, P., Kalueff, A.V., 2010. Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. Nat. Protoc. 5, 1786–1799. https://doi.org/10.1038/nprot.2010.140.

Capriello, T., Grimaldi, M.C., Cofone, R., D'Aniello, S., Ferrandino, I., 2019. Effects of aluminium and cadmium on hatching and swimming ability in developing zebrafish. Chemosphere 222, 243–249. https://doi.org/10.1016/j.chemosphere.2019.01.140.

Carotenuto, R., Capriello, T., Cofone, R., Galdiero, G., Fogliano, C., Ferrandino, I., 2020. Impact of copper in Xenopus laevis liver: histological damages and atp7b downregulation. Ecotoxicol. Environ. Saf. 30, 188–109940. https://doi.org/10.1016/j.ecoenv.2019.109940.

Chen, W.Y., John, J.A.C., Lin, C.-H., Lin, H.-F., Wu, S.-C., Lin, C.-H., Chang, C.-Y., 2004. Expression of Metallothionein gene during embryonic and early larval development in zebrafish. Aquat. Toxicol. 69, 215–227. https://doi.org/10.1016/j.aquatox.2004.05.004.

Chen, L., Ma, L., Bai, Q., Zhu, X., Zhang, J., Wei, Q., Li, D., Gao, C., Li, J., Zhang, Z., Liu, C., He, Z., Zeng, X., Zhang, A., Qu, W., Zhuang, Z., Chen, W., Xiao, Y., 2014. Heavy metal-induced metallothionein expression is regulated by specific protein phosphatase 2A complexes. J. Biol. Chem. 289 (32), 22413–22426. https://doi.org/10.1074/jbc.M114.548677.

Cho, Y.S., Choi, B.N., Kim, K.H., Kim, S.K., Kim, D.S., Bang, I.C., Nam, Y.K., 2006. Differential expression of Cu/Zn superoxide dismutase mRNA during exposures to heavy metals in rockbream (Oplegnathusfasciatus). Aquaculture 253, 667–679. https://doi.org/10.1016/j.aquaculture.2005.05.047.

Claiborne, A., 1985. Catalase Activity. Catalase Activity, pp. 283–284.

Coleman, J.G., Johnson, D.R., Stanley, J.K., Bednar, A.J., Weiss Jr., C.A., Boyd, R.E., Steevens, J.A., 2010. Assessing the fate and effects of nano aluminum oxide in the terrestrial earthworm, Eisenia fetida. Environ. Toxicol. Chem. 29 (7), 1575–1580. https://doi.org/10.1002/etc.196, 2010.

Coyle, P., Philcox, J.C., Carey, L.C., Rofe, A.M., 2002. Metallothionein: the multipurpose protein. Cell. Mol. Life Sci. 59 (4), 627–647. https://doi.org/10.1007/s00018-002-8454-2.

de Carvalho, T.S., Cardoso, P.B., Santos-Silva, M., Lima-Bastos, S., Luz, W.L., Assad, N., Kauffmann, N., Passos, A., Brasil, A., Bahia, C.P., Moraes, S., Gouveia, A.Jr., de Jesus, Oliveira., Batista, E., Oliveira, K.R., Herculano, A.M., 2019. Oxidative stress mediates anxiety-like behavior induced by high caffeine intake in zebrafish: protective effect of alpha-tocopherol. Oxid. Med. Cell. Longev. 8419810. https://doi.org/10.1155/2019/8419810.

De Smet, H., De Wachter, B., Lobinski, R., Blust, R., 2001. Dynamics of (Cd,Zn)-metallothioneins in gills, liver and kidney of common carp Cyprinus carpio during cadmium exposure. Aquat. Toxicol. 52, 269–281. https://doi.org/10.1016/S0166-445X(00)00136-3.

Deng, J., Yu, L., Liu, C., Yu, K., Shi, X., Yeung, L.W., Lam, P.K., Wu, R.S., Zhou, B., 2009. Hexabromocyclododecane-induced developmental toxicity and apoptosis in zebrafish embryos. Aquat. Toxicol. 29–36. https://doi.org/10.1016/j.aquatox.2018.03.017.

Dipp, V.R., Valles, S., Ortiz-Kerbertt, H., Suarez, J.V., Bardullas, U., 2018. Neurobehavioral alterations in zebrafish due to long-term exposure to low doses of inorganic arsenic. Zebrafish 15 (6), 575–585. https://doi.org/10.1089/zeb.2018.1627.

Domingues, I., Oliveira, R., Lourenco, J., Grisolia, C.K., Mendo, S., Soares, A.M., 2010. Biomarkers as a tool to assess effects of chromium (VI): comparison of responses in zebrafish early life stages and adults. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 152, 338–345. https://doi.org/10.1016/j.cbpc.2010.05.010.

Durak, I., Yurtarslanl, Z., Canbolat, O., Akyol, O., 1993. A methodological approach to superoxide dismutase (SOD) activity assay based on inhibition of nitroblue tetrazolium (NBT) reduction. Clin. Chim. Acta 214, 103–104. https://doi.org/10.1016/0009-8981(93)90307-p.

Egan, R.J., Bergner, C.L., Hart, P.C., Cachat, J.M., Canavello, P.R., Elegante, M.F., Elkhayat, S.I., Bartels, B.K., Tien, A.K., Tien, D.H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z., Kalueff, A.V., 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav. Brain Res. 205, 38–44. https://doi.org/10.1016/j.bbr.2009.06.022.

Engeszer, R.E., Patterson, L.B., Rao, A.A., Parichy, D.M., 2007. Zebrafish in the wild: a review of natural history and new notes from the field. Zebrafish 4, 21–40. https://doi.org/10.1089/zeb.2006.9997.

Exley, C., 2004. The pro-oxidant activity of aluminum. Free Radic. Biol. Med. 36 (3), 380–387. https://doi.org/10.1016/j.freeradbiomed.2003.11.017.

Favorito, R., Grimaldi, M.C., Coppola, M., Ferrandino, I., 2010. Effects of acute cadmium exposure on the pituitary gland of Podarcis sicula. Open Zool. J. 3, 30–36. https://doi.org/10.2174/1874336601003010030.

Favorito, R., Chiarelli, G., Grimaldi, M.C., De Bonis, S., Lancieri, M., Ferrandino, I., 2011. Bioaccumulation of cadmium and its cytotoxic effect on zebrafish brain. Chem. Ecol. 27 (2), 39–46. https://doi.org/10.1080/02757540.2011.625937.

Favorito, R., Monaco, A., Grimaldi, M.C., Ferrandino, I., 2017. Effects of cadmium on the glial architecture in lizard brain. Eur. J. Histochem. 61, 2734. https://doi.org/10.4081/ejh.2017.2734.

Félix, L.M., Vidal, A.M., Serafim, C., Valentim, A.M., Antunes, L.M., Campos, S., Matos, M., Monteiro, S.M., Coimbra, A.M., 2016. Ketamine-induced oxidative stress at different developmental stages of zebrafish (Danio rerio) Embryos. RSC Adv. 6 (66), 61254–61266. https://doi.org/10.1039/c6ra08298j.

Félix, L.M., Vidal, A.M., Serafim, C., Valentim, A.M., Antunes, L.M., Monteiro, S.M., Matos, M., Ana, M., Coimbra, A.M., 2018. Ketamine induction of p53-dependent apoptosis and oxidative stress in zebrafish (Danio rerio) embryos. Chemosphere 201, 730–739. https://doi.org/10.1016/j.chemosphere.2018.03.049.

Ferrandino, I., Grimaldi, M.C., 2008. Ultrastructural study of the pituicytes in the pituitary gland of the teleost Diplodus sargus. Brain Res. Bull. 75 (1), 133–137. https://doi.org/10.1016/j.brainresbull.2007.08.006.

Ferrandino, I., Favorito, R., Annunziata, M., Grimaldi, M.C., 2009. Cadmium induces apoptosis in the pituitary gland of Podarcis sicula. Ann. N. Y. Acad. Sci. 1163, 386–388. https://doi.org/10.1111/j.1749-6632.2008.03667.x.

Flora, S.J., Mehta, A., Satsangi, K., Kannan, G.M., Gupta, M., 2003. Aluminum-induced oxidative stress in rat brain: response to combined administration of citric acid and HEDTA. Comp. Biochem. Physiol. Part C 134, 19–328. https://doi.org/10.1016/s1532-0456(02)00269-7.

Forsido, T.T., McCrindle, R.I., Maree, J., Monyatsi, L., 2020. Removal of Al, Ba and Mg from industrial wastewater using EAFDS and lime. Appl. Water Sci. 10, 157. https://doi.org/10.1007/s13201-020-01234-y.

Franco-Restrepo, J.E., Forero, D.A., Vargas, R.A., 2019. A review of freely available, open-source software for the automated analysis of the behavior of adult zebrafish. Zebrafish 16 (3), 223–232. https://doi.org/10.1089/zeb.2018.1662.

Frasco, M.F., Guilhermino, L., 2002. Effects of dimethoate and beta-naphthoflavone on selected biomarkers of Poecilia reticulata. Fish Physiol. Biochem. 26, 149–156. https://doi.org/10.1023/A:1025457831923.

Gerlai, R., Ahmad, F., Prajapati, S., 2008. Differences in acute alcohol-induced behavioral responses among zebrafish populations. Alcohol. Clin. Exp. Res. 32, 1763–1773. https://doi.org/10.1111/j.1530-0277.2008.00761.x.

Ghorbel, I., Chaabane, M., Elwej, A., Boudawara, O., Abdelhedi, S., Jamoussi, K., Boudawara, T., Zeghal, N., 2016. Expression of metallothioneins I and II related to oxidative stress in the liver of aluminium-treated rats. Arch. Physiol. Biochem. 122 (4), 214–222. https://doi.org/10.1080/13813455.2016.1187176.

Ghoshal, K., Jacob, S.T., 2001. Regulation of metallothionein gene expression. Prog. Nucleic Acid Res. Mol. Biol. 66, 357–384. https://doi.org/10.1016/s0079-6603(00)66034-8.

Habig, W.H., Jakoby, W.B., 1981. Assays for differentiation of glutathione S-Transferases. Methods Enzymol 77, 398–405. https://doi.org/10.1016/s0076-6879(81)77053-8.

Harvey, A.L., Kind, K.L., Thompson, J.G., 2002. REDOX regulation of early embryo development. Reproduction 123, 479–486. https://doi.org/10.1530/rep.0.1230479.

Ighodaro, O.M., Akinloye, O.A., 2018. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their fundamental role in the entire antioxidant defence grid. Alexandria J. Med. 54 (4), 287–293. https://doi.org/10.1016/j.ajme.2017.09.001.

Jihen el, H., Fatima, H., Nouha, A., Baati, T., Imed, M., Abdelhamid, K., 2010. Cadmium retention increase: a probable key mechanism of the protective effect of zinc on cadmium-induced toxicity in the kidney. Toxicol. Lett. 196 (2), 104–109. https://doi.org/10.1016/j.toxlet.2010.04.006.

Jijie, R., Solcan, G., Nicoara, M., Micu, D., Strungaru, S., 2020. Antagonistic effects in zebrafish (Danio rerio) behavior and oxidative stress induced by toxic metals and deltamethrin acute exposure. Sci. Total Environ. 698, 134299. https://doi.org/10.1016/j.scitotenv.2019.134299.

Jin, Y., Liu, Z., Liu, F., Ye, Y., Peng, T., Fu, Z., 2015. Embryonic exposure to cadmium (II) and chromium (VI) induce behavioral alterations, oxidative stress and immunotoxicity in zebrafish (Danio rerio). Neurotoxicol. Teratol. 48, 9–17. https://doi.org/10.1016/j.ntt.2015.01.002.

Kalueff, A.V., Gebhardt, M., Stewart, A.M., Cachat, J.M., Brimmer, M., Chawla, J.S., Craddock, C., Kyzar, E.J., Roth, A., Landsman, S., Gaikwad, S., Robinson, K., Baatrup, E., Tierney, K., Shamchuk, A., Norton, W., Miller, N., Nicolson, T., Braubach, O., Gilman, C.P., Pittman, J., Rosemberg, D.B., Gerlai, R., Echevarria, D., Lamb, E., Neuhauss, S.C., Weng, W., Bally-Cuif, L., Schneider, H., Zebrafish Neuroscience Research Consortium, 2013. Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. Zebrafish 10 (1), 70–86. https://doi.org/10.1089/zeb.2012.0861.

Kent, M.L., Buchner, C., Barton, C., Tanguay, R.L., 2014. Toxicity of chlorine to zebrafish embryos. Dis. Aquat. Organ. 107 (3), 235–240. https://doi.org/10.3354/dao02683.

Kim, J.W., Dang, C.V., 2006. Cancer's molecular sweet tooth and the Warburg effect. Cancer Res. 66 (18), 8927–8930. https://doi.org/10.1158/0008-5472.CAN-06-1501.

Kishido, T., Unno, K., Yoshida, H., Choba, D., Fukutomi, R., Asahina, S., Iguchi, K., Oku, N., Hoshino, M., 2007. Decline in glutathione peroxidase activity is a reason for brain senescence: consumption of green tea catechin prevents the decline in its activity and protein oxidative damage in ageing mouse brain. Biogerontology 8 (4), 423–430. https://doi.org/10.1007/s10522-007-9085-7.

Kumar, S., 1999. Aluminium-induced biphasic effect. Med. Hypothesis. 52, 557–559. https://doi.org/10.1054/mehy.1997.0693.

Lança, M.J., Machado, M., Ferreira, A.F., Quintella, B.R., de Almeida, P.R., 2015. Structural lipid changes and Na(+)/K(+)-ATPase activity of gill cells' basolateral membranes during saltwater acclimation in sea lamprey (Petromyzon marinus, L.) juveniles. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 189, 67–75. https://doi.org/10.1016/j.cbpa.2015.07.018.

Lanzarin, G.A.B., Félix, L.M., Santos, D., Venâncio, C.A.S., Monteiro, S.M., 2019. Dose-dependent effects of a glyphosate commercial formulation - Roundup® UltraMax - on the early zebrafish embryogenesis. Chemosphere 223, 514–522. https://doi.org/10.1016/j.chemosphere.2019.02.071.

Larose, C., Canuel, R., Lucotte, M., Di Giulio, R.T., 2008. Toxicological effects of methylmercury on walleye (Sander vitreus) and perch (Perca flavescens) fromlakes of the boreal forest. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 147 (2), 139–149. https://doi.org/10.1016/j.cbpc.2007.09.002.

Lau, B.Y., Mathur, P., Gould, G.G., Guo, S., 2011. Identification of a brain center whose activity discriminates a choice behavior in zebrafish. Proc. Natl. Acad. Sci. U. S. A. 108 (6), 2581–2586. https://doi.org/10.1073/pnas.1018275108.

Lawrence, G.B., Sutherland, J.W., Boylen, C.W., Nierzwicki-Bauer, S.W., Momen, B., Baldigo, B.P., Simonin, H.A., 2007. Acid rain effects on aluminum mobilization clarified by inclusion of strong organic acids. Environ. Sci. Technol. 41 (1), 93–98. https://doi.org/10.1021/es061437v.

Levine, R.L., 2002. Carbonyl modified proteins in cellular regulation, aging, and disease. Free Radic. Biol. Med. 32 (9), 790–796. https://doi.org/10.1016/s0891-5849(02)00765-7.

Li, S., Yan, T., Yang, J.Q., Oberley, T.D., Oberley, L.W., 2000. The role of cellular glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese superoxide dismutase. Cancer Res. 60 (14), 3927–3939.

Linde, A.R., Garcia-Vazquez, E., 2006. A simple assay to quantify metallothionein helps to learn about bioindicators and environmental health. Biochem. Mol. Biol. Educ. 34 (5), 360–363. https://doi.org/10.1002/bmb.2006.494034052653.

Lushchak, V.I., 2014. Free radicals, reactive oxygen species, oxidative stress and its classification. Chem. Biol. Interact. 224, 164–175. https://doi.org/10.1016/j.cbi.2014.10.016.

Maheswari, S.L., Venkatakrishna Murali, R., Balaji, R., 2014. Aluminium induced cholinotoxicity in zebra fish brain - A sequel of oxidative stress. IJAR 2 (2), 322–335. ISSN 2320-5407.

Mandl, P., Kiss, J.P., 2007. Role of presynaptic nicotinic acetylcholine receptors in the regulation of gastrointestinal motility. Brain Res. Bull. 72 (4–6), 194–200. https://doi.org/10.1016/j.brainresbull.2007.02.005, 30.

Massarsky, A., Kozal, J.S., Di Giulio, R.T., 2017. Glutathione and zebrafish: old assays to address a current issue. Chemosphere 168, 707–715. https://doi.org/10.1016/j.chemosphere.2016.11.004.

Meister, A., 1994. Glutathione, ascorbate, and cellular protection. Cancer Res. 54, 1969s–1975s.

Mesole, S.B., Alfred, O.O., Yusuf, U.A., Lukubi, L., Ndhlovu, D., 2020. Apoptotic inducement of neuronal cells by aluminium chloride and the neuroprotective effect of eugenol in wistar rats. Oxid. Med. Cell. Longev. 8425643. https://doi.org/10.1155/2020/8425643.

Misra, S., Niyogi, S., 2009. Selenite causes cytotoxicity in rainbow trout (Oncorhynchus mykiss) hepatocytes by inducing oxidative stress. Toxicol. In Vitro 23, 1249–1258. https://doi.org/10.1016/j.tiv.2009.07.03.

Monaco, A., Grimaldi, M.C., Ferrandino, I., 2016. Neuroglial alterations in the zebrafish brain exposed to cadmium chloride. J. Appl. Toxicol. 36 (12), 1629–1638. https://doi.org/10.1002/jat.3328.

Monaco, A., Capriello, T., Grimaldi, M.C., Schiano, V., Ferrandino, I., 2017a. Neurodegeneration in zebrafish embryos and adults after cadmium exposure. Eur. J. Histochem. 61 (4) https://doi.org/10.4081/ejh.2017.2833, 19PMC5656802.

Monaco, A., Grimaldi, M.C., Ferrandino, I., 2017b. Aluminium chloride-induced toxicity in zebrafish larvae. J. Fish Dis. 40 (5), 629–635. https://doi.org/10.1111/jfd.12544.

Motta, C.M., Simoniello, P., Arena, C., Capriello, T., Panzuto, R., Vitale, E., Agnisola, C., Tizzano, M., Avallone, B., Ferrandino, I., 2019. Effects of four food dyes on development of three model species, *Cucumis sativus*, *Artemia salina* and *Danio rerio*: assessment of potential risk for the environment. Environ. Pollut. 253, 1126–1135. https://doi.org/10.1016/j.envpol.2019.06.018.

Mujika, J.I., Rezabal, E., Mercero, J.M., Ruipérez, F., Costa, D., Ugalde, J.M., Lopez, M., 2014. Aluminium in biological environments: a computational approach. Comput. Struct. Biotechnol. J. 9 https://doi.org/10.5936/csbj.201403002 e201403002.

Nehru, B., Anand, P., 2005. Oxidative damage following chronic aluminium exposure in adult and pup rat brains. J. Trace Elem. Med. Biol. 19 (2-3), 203–208. https://doi.org/10.1016/j.jtemb.2005.09.004.

Newman, M., Verdile, G., Martins, R.N., Lardelli, M., 2011. Zebrafish as a tool in Alzheimer's disease research. Biochim. Biophys. Acta 1812 (3), 346–352. https://doi.org/10.1016/j.bbadis.2010.09.012.

Oliveira, M., Teixeira, J.C., Vasconcelos-Nobrega, C., Félix, L.M., Sardao, V.A., Colaco, A. A., Oliveira, P.A., Peixoto, F.P., 2013. Mitochondrial and liver oxidative stress alterations induced by N-butyl-N-(4-hydroxybutyl)nitrosamine: relevance for hepatotoxicity. J. Appl. Toxicol. 33, 434–443. https://doi.org/10.1002/jat.1763.

Oliveira, R., Grisolia, C.K., Monteiro, M.S., Soares, A.M., Domingues, I., 2016. Multilevel assessment of ivermectin effects using different zebrafish life stages. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 187, 50–61. https://doi.org/10.1016/j.cbpc.2016.04.004.

Orger, M.B., de Polavieja, G.G., 2017. Zebrafish behavior: opportunities and challenges. Annu. Rev. Neurosci. 40, 125–147. https://doi.org/10.1146/annurev-neuro-071714-033857.

Pan, Y.X., Luo, Z., Zhuo, M.Q., Wei, C.C., Chen, G.H., Song, Y.F., 2018. Oxidative stress and mitochondrial dysfunction mediated Cd-induced hepatic lipid accumulation in zebrafish Danio rerio. Aquat. Toxicol. 199, 12–20. https://doi.org/10.1016/j.aquatox.2018.03.017.

Park, J.D., Liu, Y., Klaassen, C.D., 2001. Protective effect of metallothionein against the toxicity of cadmium and other metals(1). Toxicology 163 (2-3), 93–100. https://doi.org/10.1016/s0300-483x(01)00375-4.

Peng, J.H., Xu, Z., Parker, J., Friedlander, E., Tang, J., Melethil, S., 1992. Aluminum-induced acute cholinergic neurotoxicity in rat. Mol. Chem. Neuropathol. 17 (1), 79–89. https://doi.org/10.1007/bf03159983.

Pham, M., Raymond, J., Hester, J.D., Kyzar, E.J., Gaikwad, S., Bruce, I., Fryar, C., Chanin, S., Enriquez, J., Bagawandoss, S., Zapolsky, I., Green, J., Stewart, A.M., Robison, B.D., Kalueff, A.V., 2012. Assessing social behavior phenotypes in adult Zebrafish: shoaling, social preference, and mirror biting tests. Zebrafish Protocols Neurobehav. Res. 231–246. https://doi.org/10.1007/978-1-61779-597-8_17.

Pilehvar, A., Town, R.M., Blust, R., 2020. The effect of copper on behaviour, memory, and associative learning ability of zebrafish (Danio rerio). Ecotoxicol. Environ. Saf. 188–109900. https://doi.org/10.1016/j.ecoenv.2019.109900.

Quintana-Villamandos, B., González, M.C., Delgado-Martos, M.J., Condezo-Hoyos, L., Böger, R.H., Lüneburg, N., Pazó-Sayós, L., Gutiérrez-Arzapalo, P.Y., Delgado-Baeza, E., 2016. Short-term esmolol attenuates remodeling of the thoracic aorta in hypertensive rats by decreasing concentrations of ADMA down-regulated by oxidative stress. Eur. J. Pharmacol. 791, 502–509. https://doi.org/10.1016/j.ejphar.2016.09.020.

Riehl, R., Kyzar, E., Allain, A., Green, J., Hook, M., Monnig, L., Rhymes, K., Roth, A., Pham, M., Razavi, R., Dileo, J., Gaikwad, S., Hart, P., Kalueff, A.V., 2011. Behavioral and physiological effects of acute ketamine exposure in adult zebrafish. Neurotoxicol. Teratol. 33 (6), 658–667. https://doi.org/10.1016/j.ntt.2011.05.011.

Rodriguez, A., Zhang, H., Klaminder, J., Brodin, T., Andersson, P.L., Andersson, M., 2018. ToxTrac: a fast and robust software for tracking organisms. MEE 9 (3), 460–464. https://doi.org/10.1111/2041-210X.12874.

Rodriguez-Fuentes, G., Rubio-Escalante, F.J., Norena-Barroso, E., Escalante-Herrera, K. S., Schlenk, D., 2015. Impacts of oxidative stress on acetylcholinesterase transcription, and activity in embryos of zebrafish (Danio rerio) following Chlorpyrifos exposure. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 172-173, 19–25. https://doi.org/10.1016/j.cbpc.2015.04.003.

Ruiter, S., Sippel, J., Bouwmeester, M.C., Lommelaars, T., Beekhof, P., Hodemaekers, H. M., Bakker, F., van den Brandhof, E.J., Pennings, J.L., van der Ven, L.T., 2016. Programmed effects in Neurobehavior and antioxidative physiology in zebrafish embryonically exposed to cadmium: observations and hypothesized adverse outcome pathway framework. Int. J. Mol. Sci. 17 (11) https://doi.org/10.3390/ijms17111830.

Sager, J.J., Bai, Q., Burton, E.A., 2010. Transgenic zebrafish models of neurodegenerative diseases. Brain Struct. Funct. 214 (2–3), 285–302. https://doi.org/10.1007/s00429-009-0237-1.

Sánchez-Iglesias, S., Méndez-Alvarez, E., Iglesias-González, J., Muñoz-Patiño, A., Sánchez-Sellero, I., Labandeira-García, J.L., Soto-Otero, R., 2009. Brain oxidative stress and selective behaviour of aluminium in specific areas of rat brain: potential

effects in a 6-OHDA-induced model of Parkinson's disease. J. Neurochem. 109 (3), 879–888. https://doi.org/10.1111/j.1471-4159.2009.06019.x.
Santos, D., Matos, M., Coimbra, A.M., 2014. Developmental toxicity of endocrine disruptors in early life stages of zebrafish, a genetic and embryogenesis study. Neurotoxicol. Teratol. 46, 18–25. https://doi.org/10.1016/j.ntt.2014.08.002.
Santos, D., Luzio, A., Coimbra, A.M., 2017. Zebrafish sex differentiation and gonad development: a review on the impact of environmental factors. Aquat. Toxicol. 191, 141–163. https://doi.org/10.1016/j.aquatox.2017.08.005.
Santos, D., Vieira, R., Luzio, A., Félix, L., 2018. Chapter five - zebrafish early life stages for toxicological screening: insights from molecular and biochemical markers. Adv. Mol. Toxicol. 12, 151–179. https://doi.org/10.1016/B978-0-444-64199-1.00007-5.
Senger, M.R., Seibt, K.J., Cordenonzi, G., Dutra Dias, R., Bogo, M.R., Bonan, C.D., 2011. Aluminum exposure alters behavioral parameters and increases acetylcholinesterase activity in zebrafish (Danio rerio) brain. Cell Biol. Toxicol. 27 (3), 199–205. https://doi.org/10.1007/s10565-011-9181-y.
Silva Santos, N., Oliveira, R., Lisboa, C.A., Pinto, J.M.E., Sousa-Moura, D., Camargo, N.S., Perillo, V., Oliveira, M., Grisolia, C.K., Domingues, I., 2018. Chronic effects of carbamazepine on zebrafish: behavioral, reproductive and biochemical endpoints. Ecotoxicol. Environ. Saf. 164, 297–304. https://doi.org/10.1016/j.ecoenv.2018.08.01.
Singla, N., Dhawan, D.K., 2014. Zinc modulates aluminium-induced oxidative stress and cellular injury in rat brain. Metallomics 6 (10), 1941–1950. https://doi.org/10.1039/c4mt00097h.
Soreq, H., Seidman, S., 2001. Acetylcholinesterase–new roles for an old actor. Nat. Rev. Neurosci. 2 (4), 294–302. https://doi.org/10.1038/35067589.
Srikanthan, K., Shapiro, J.I., Sodhi, K., 2016. The role of Na/K-ATPase signaling in oxidative stress related to obesity and cardiovascular disease. Molecules 21 (9), 1172. https://doi.org/10.3390/molecules21091172.
Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy metals toxicity and the environment. EXS 101, 133–164. https://doi.org/10.1007/978-3-7643-8340-4_6.
Tönnies, E., Trushina, E., 2017. Oxidative stress, synaptic dysfunction, and alzheimer's disease. J. Alzheimers Dis. 57 (4), 1105–1121. https://doi.org/10.3233/JAD-161088.
Tseng, Y.C., Chen, R.D., Lucassen, M., Schmidt, M.M., Dringen, R., Abele, D., PuHwang, P., 2011. Exploring uncoupling proteins and antioxidant mechanisms under acute cold exposure in brains of fish. PLoS One 6 (3), e18180. https://doi.org/10.1371/journal.pone.0018180.
Valvona, C.J., Fillmore, H.L., Nunn, P.B., Pilkington, G.J., 2016. The regulation and function of lactate dehydrogenase a: therapeutic potential in brain tumor. Brain Pathol. 26 (1), 3–17. https://doi.org/10.1111/bpa.12299.
Van Campenhout, K., Infante, H.G., Hoff, P.T., Moens, L., Goemans, G., Belpaire, C., Adams, F., Blust, R., Bervoets, L., 2010. Cytosolic distribution of Cd, Cu and Zn, and metallothionein levels in relation to physiological changes in gibel carp (Carassius auratus gibelio) from metal-impacted habitats. Ecotoxicol. Environ. Saf. 73, 296–305. https://doi.org/10.1016/j.ecoenv.2009.10.007.
Wallin, B., Rosengren, B., Shertzer, H.G., Camejo, G., 1993. Lipoprotein oxidation and measurement of thiobarbituric acid reacting substances formation in a single microtiter plate: its use for evaluation of antioxidants. Anal. Biochem. 208, 10–15. https://doi.org/10.1006/abio.1993.1002.
Wang, T., Wang, Q., Song, R., Zhang, Y., Yang, J., Wang, Y., Yuan, Y., Bian, J., Liu, X., Gu, J., Zhu, J., Liu, Z., 2016. Cadmium induced inhibition of autophagy is associated with microtubule disruption and mitochondrial dysfunction in primary rat cerebral cortical neurons. Neurotoxicol. Teratol. 53, 11–18. https://doi.org/10.1016/j.ntt.2015.11.007.
Way, G.P., Ruhl, N., Snekser, J.L., Kiesel, A.L., McRobert, S.P., 2015. A comparison of methodologies to test aggression in zebrafish. Zebrafish 12 (2), 144–151. https://doi.org/10.1089/zeb.2014.1025.
Westerfield, M., 2000. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), fourth ed. University of Oregon Press, Eugene.
WHO Guidelines for Drinking-water Quality, 2021. Aluminium in drinking-water. WHO/HSE/WSH/10.01/13.
World Health Organization, Geneva, Switzerland, 1997. Environmental Health Criteria for Aluminium. Environmental Health Criteria No.194. pp.xx + 282 pp. ref.many. ISSN: 0250-863X.
Yang, M., Qiu, W., Chen, J., Zhan, J., Pan, C., Lei, X., Wu, M., 2014. Growth inhibition and coordinated physiological regulation of zebrafish (Danio rerio) embryos upon sublethal exposure to antidepressant amitriptyline. Aquat. Toxicol. 151, 68–76. https://doi.org/10.1016/j.aquatox.2013.12.029.
Yousef, M.I., 2004. Aluminium-induced changes in hemato-biochemical parameters, lipid peroxidation and enzyme activities of male rabbits: protective role of ascorbic acid. Toxicology 199 (1), 47–57. https://doi.org/10.1016/j.tox.2004.02.014.
Zatta, P., Ibn-Lkhayat-Idrissi, M., Zambenedetti, P., Kilyen, M., Kiss, T., 2002. In vivo and in vitro effects of aluminum on the activity of mouse brain acetylcholinesterase. Brain Res. Bull. 59 (1), 41–45. https://doi.org/10.1016/s0361-9230(02)00836-5.
Zenki, K.C., Mussulini, B.H., Rico, E.P., de Oliveira, D.L., Rosemberg, D.B., 2014. Effects of ethanol and acetaldehyde in zebrafish brain structures: an in vitro approach on glutamate uptake and on toxicity-related parameters. Toxicol. In Vitro 28 (5), 822–828. https://doi.org/10.1016/j.tiv.2014.03.008.
Zhang, J., Lyu, Z., Shao, S., Fang fang Li, F., Yang, S., Song, W., Li, W., Li, S., 2016. Effects of aluminum toxicity induced by acid deposition on pine forest ecosystem in Longli of Guizhou Province, Southwestern China. Chin. Geogr. Sci. 26, 495–507. https://doi.org/10.1007/s11769-015-0763-0.

Aluminium exposure in zebrafish leads to neurodegeneration and changes in the gene-expression of markers involved to Parkinsonism

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Abstract

Aluminium, despite being it is extremely diffuse in the world, it is a non-essential metal to human metabolism. It is known to have toxic effects on a variety of organs including the brain and it is considered an etiological factor in neurodegenerative diseases like Parkinson and Alzheimer diseases. However, the molecular mechanisms by which aluminium exerts its neurotoxic effects are still not completely understood. The zebrafish is a useful animal model both for ecotoxicology studies and for exploring neurodegenerative diseases, since the overall anatomical organization of the central and peripheral nervous system is relatively conserved in zebrafish and mammals. Adult zebrafish were exposed to 11 mg/L of Al for 10, 15 and 20 days and the neurotoxic effects of aluminium were analysed by histological, immunohistochemical and molecular evaluations. Precisely, histological stainings allowed to evaluate the morphology of the brain' parenchyma, myelin alteration and activations of neurodegenerative processes. The immunohistochemistry experiments performed with an antibody anti-GFAP, marker of glial cells, were applied on sections to observe the alteration of the glia. Finally, the evaluation of markers involved in the parkinsonism by RT-qPCR, were assessed to better understand the role of aluminium in the regulation of genes related to Parkinson's neurodegenerative disease. The collected data showed a fairly constant trend with greater effects recorded in the early stages of exposure, 10 and 15 days, and an improvement/stabilization of

conditions for longer exposure times, 20 days. Overall, the data confirm the neurotoxicity induced by aluminium and inform about its involvement in neurodegenerative processes.

Keyword: Brain, Gene expression, Glial fibrillary acidic protein, Histology, Immunohistochemistry, Myelin.

1. Introduction

Aluminium (Al) is among the naturally most widespread metals in the environment, and its quantity is further increased by anthropogenic sources (Igbokwe et al., 2019). The Al ion has no physiological role in metabolic processes (Exley and House, 2011) but it can be a toxicant agent to humans and animals (Becaria et al., 2002). Al was considered unsafe to humans after the discovery of increased levels of Al in brain tissues of patients with encephalopathy (Alfrey and Solomons, 1976). Al accumulation in mammalian tissues was associated with various pathologic effects: pulmonary lesions (Kongerud and Søyseth, 2014), bone abnormalities (Klein, 2019), and neurologic disorders (Colomina and Peris-Sampedro, 2017; Morris et al., 2017). Indeed, Al can rapidly enter the brain, extracellular fluid and the cerebrospinal fluid (Krewski et al., 2007) and its accumulation in the brain has been associated with neurodegenerative diseases such Alzheimer's disease (AD), Parkinson's disease (PD), amyotropic lateral sclerosis (SLA) (Arain et al., 2015; Bjorklund et al., 2018; Jones et al., 2017; Kawahara and Kato-Negishi, 2011). Al exposure can promote oxidative stress in the nervous tissue, inducing neurodegeneration and neuronal necrosis and, which constitute the basis for the neurological diseases associated with Al toxicity (Bondy, 2016; Exley, 2006). For this reasons, particular attention has been placed on the neurotoxic effect of Al, having already evaluated in a previous study the ability of Al to alter the swimming activity, the behaviour and the oxidative state of the zebrafish brain (Capriello et al., 2021). The choice to use *Danio rerio*, also called zebrafish, as a model organism was not accidental, since in addition to being known as an excellent model for ecotoxicological studies (Bambino and Chu, 2017; Favorito et al., 2011), it has become an important animal model for exploring neurodegenerative diseases because its nervous system is simple, but it

shares functional and anatomical similarities with the human nervous system (Babin et al., 2014). In this case, the adult zebrafish were exposed to 11 mg/L of Al using 0.1g/L of $AlCl_3 \cdot 6H_2O$ dissolved in water, as a source. The concentration, also used previously, (Capriello et al., 2021) can be reconnected to that found in polluting waters (ATSDR, 2008). The effects induced by Al on the brain were analysed at three different exposure times: 10, 15 and 20 days. Precisely, evaluations were carried out for the quantization of Al inside the brain after the different exposure times, but also histological analyses that made it possible to visualize the effect induced on the brain parenchyma, the alteration of myelin and the induced neurodegeneration. Finally, the expression of the Glial fibrillary acidic protein (GFAP), and the gene expression of the markers involved in Parkinsonism were assessed. GFAP one of the most abundant cytoskeletal constituents of glial cells, crucial for their functionality and integrity (Middeldorp and Hol, 2011), and its expression was altered in the zebrafish brain after exposure to other metals such as cadmium (Monaco et al., 2016). Furthermore, it plays an important role in the development and progression of neurodegenerative diseases (Middeldorp and Hol, 2011; Schiffer et al., 1996). Instead, the genes linked to Parkinsonism were chosen because unlike Alzheimer's disease, the connection between exposure to Al and this disease is little studied, although several examples of connection between them have been reported in the literature (Bondy, 2014). Overall, the analysed data in this study want to add an important step in the study of the correlation Al exposure-neurotoxicity, in order to better understand the effect of this metal on human health.

2. Material and methods

2.1 Zebrafish housing

Adult zebrafish were maintained at the Department of Biology of the University of Naples “Federico II”. Fish were housed in rectangular tanks under 12 h light:12 h dark photoperiod, at a temperature of 28 ◦C, and fed twice a day with a commercial diet (TetraMin Tropical Flake Fish®) supplemented with *Artemia* sp. nauplii (Westerfield, 2000). All used protocols were in accordance with the

guidelines dictated by European regulations on animal of welfare (Directive 2010/63/EU) and approved by the Italian Ministry of Health (permits 147/2019-PR).

2.2 Preparation of treatment solution and chemical test

The treatment solution (11 mg/L of Al) was prepared by dissolving 2.5 g of $AlCl_3 \cdot 6H_2O$ (Carlo Erba, Italy; CAS no. 7784-13-6) in 25 L of water (1/3 bidistilled water, 2/3 tap water), directly into the treatment tank like in previous study (Capriello et al., 2021). Moreover, Al concentration in the treated solution was checked on samples taken randomly during the treatment period, by MP-AES (Microwave Plasma-Atomic Emission Spectrometry), to confirm the accuracy of treatment. Al concentration mentioned during the text refer to the measured concentration.

2.3 Al exposure and samples collection

Three groups with each 12 healthy zebrafish (6 male and 6 female), between 6-12 months of life, were placed in a 25 L tanks and exposed respectively for 10, 15 and 20 days to 0.1 g/L of $AlCl_3$ solution (11 mg/L Al). Another group of 12 fish (6 male and 6 female), exposed only to tank water (1/3 bidistilled water, 2/3 tap water), was used as a control group. A biological triplicate was performed for each treatment. The treatment solution was totally renewed and the food debris were removed once a day. During fish treatment, water parameters were monitored daily and kept in the following ranges: temperature 28°C and pH: 6.5 to 7.5. After experiments, the organisms were euthanized with an overdose of ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma Aldrich, Germany) and the brains were collected and processed to perform the several analyses. Four brains (2 male and 2 female), from the replicates of each experimental group, were used for each analysis performed. Precisely 4 brains were used for to assessment of Al concentration, 4 brain were selected for histological analyses and others for RT-qPCR analyses.

2.4 Assessment of Al concentration

To determine the total Al content in zebrafish brains, they were taken and singularly frozen at −80°C, rapidly. Tissue samples was then digested in 250 µL of HNO_3 u.p. and kept in a thermostatic bath at about 90°C for 1 hour (h). Accumulated Al was quantified by Inductively coupled plasma mass spectrometry (ICP-MS). The results are expressed as µg of metal accumulated in a brain.

2.5 Histology

All the brains were performed with standard histological protocols for optical microscopy. The samples were fixed in Bouin solution for 48 h to preserve the protoplasmic structure, dehydrated and included in paraffin. Sagittal sections of 5 µm, deparaffinized and hydrated, were stained by Hematoxylin-Eosin, Methasol fast blue, Fluoro-Jade B and by the ABC immunohistochemistry technique. At the end, all images were acquired with a Kontron Electronic Imaging System KS300 (Zeiss, Germany).

2.5.1 Haematoxylin-Eosin and Methasol fast blue staining

For the evaluation of general morphology, the section was performed using a combination of two histological stains in succession: haematoxylin for 5 min and eosin acidified for 2 min (Favorito et al., 2017). At the end, the slides were dehydrated and mounted in resin for microscopic examination.

The staining Methasol fast blue, MFB, (Gurr ltd, London, England) was used to detect any demyelination in brain tissue (Monaco et al., 2016). Once the sections had been rehydrated, they were immersed in 1% of MFB for 2 h at 58 ° C, and differentiated by exposure to ethanol 95 °, water and 0.05% lithium carbonate. The slides were then placed in haematoxylin for 30 s to counterstain the nuclei, dehydrated and mounted in resin.

2.5.2 Histochemical staining by Fluoro-Jade B

To evaluate the degeneration of neurons the sections were immersed in a solution of Fluoro-Jade B (AG310 EMD, Millipore, Billerica, MA, USA), an anionic fluorochrome derived from

fluorescein. Precisely, the brain sections rehydrated were incubated for 1 h at 4°C with Fluoro-Jade B, gently shaken in the dark with an orbital shaker (Monaco et al., 2017a). Then the slides were dried overnight at room temperature. The next day the sections were clarified and mounted. The images were acquired by microscope (Zeiss, Germany) setting the FITC channel and the number of fluorescent cells was quantized in 0.1 mm^2 of brain tissue with the use of ImageJ software (Image Analysis Software, Rasband, NIH).

2.5.3 Immunohistochemistry

The immunohistochemical experiments were performed on sagittal sections of the brain using the rabbit polyclonal anti-GFAP 173002 (1:1000; Synaptic Systems, Germany). The revelation was performed using the HRP/DAB (ABC) Detection IHC Kit (ab64261, abcam, United Kingdom). The sections were counterstained by immersion in haematoxylin solution for 30 s, dehydrated and mounted in resin. Antibody specificity was assessed by omitting on some sections the primary antibody (Favorito et al., 2017; Monaco et al., 2016).

2.6 RNA extraction and qPCR analysis

Total RNA from brains of control and treated with Al for 10, 15 and 20 days were extracted using TRI reagent (SIGMA-Aldrich®, USA), according to the manufacturer's instructions. The concentration and purity of the RNA samples were assessed using a NanoDrop® 1000 spectrophotometer (Thermo Fisher, USA). The cDNA was synthesized from 1 µg of total RNA using QuantiTeck® Reverse Trascription Kit 200 (Qiagen, Germany). RT-qPCR was performed with SYBR Green assay (Applied Biosystems, USA) using AriaMx Real-time PCR System (Agilent Technologies, USA).

The mRNA level was analysed for 10 different genes involved in Parkinsonism, using respective gene specific primers (Table 1) in triplicates. The primers, previously described by Sarath Babu et al., (2016), were synthesized by Eurofins Genomics (Japan).

The PCR conditions included a denaturation step (95 °C for 10 min), 40 cycles of amplification and quantification (95 °C for 15 s, 60 °C for 1 min), followed by melt curve analysis. The relative gene expression levels were analysed from the Ct value, normalized to the *β-ACTIN* reference gene and calculated by the $2^{-\Delta\Delta Ct}$ method.

Table 1. List of genes and its forward and reverse primers used for RT-PCR

Gene	Symbol	Forward primer	Reverse primer
β-Actin	*ACT*	CATCCATCGTTCACAGGAAGTG	TGGTCGTTCGTTTGAATCTCAT
B-Synuclein	*SYNB*	AAC AGT GGC TGA GAA GAC GAA G	CTG GTT GTC GTC GTA TGT CTG T
Synapsin 2b	*SYN2B*	TTC GAC AGC ATG CCT ACA GTA T	TGA TCA ACC AGA GGG AAC TTT T
Synuclein, gamma a	*SNCGA*	ATG CAC TGA AGA AGG GAT TCT C	AGA TTT GCC TGG TCA GTT GTT T
Synuclein, gamma b	*SNCGB*	GAC TAA AGC TGG GGT TGA AGA G	CGT TCT CCA GTC CCT CTA CTG T
pTEN induced putative kinase1	*PINK1*	GGC TAC AGG CTG GAA GAT TAT G	AAG CTT GGT GAA GCA GAA AGT C
Parkin RBR E3 ubiquitin protein Ligase	*PARK2*	ACA GAC ATC ATG ACT CCA GTG C	ACA CGG AAA TGA TGA ACC TCT T
Parkinson protein 7 (DJ1) PARK7	*PARK7*	CAT ATG AAC ATG GGT TCT GCT G	GAA AGA GGT GTT GAA GGA CCA G
Park11	*GIGYF2*	ATG AGG TGC ACG ATT ATG TAC G	GTT GTT GAA GCA GGA CTG ACT G
Coiled-coil-helix-coiled-coil-helix domain containing 2	*CHCHD2*	TGT CGG ACA CAC AAT AGG TCA C	CAT ATG AAC ATG GGT TCT GCT G
Septin 3	*SEPT3*	CTT GGA TAC ATC GGC ATA GAC A	GGA ATC TTC TCA TCA CGA CTC C

2.7 Statistical analysis

Statistically significant differences among treatments were assessed by one-way analysis of variance ANOVA followed by Tukey's pairwise comparison tests for variables with normal distribution with the aid of GraphPad Prism 9 (GraphPad Software, La Jolla California USA, www.graphpad.com). Before hypothesis testing, normality and homogeneity of the data were confirmed by Shapiro-Wilk

and Brown-Forsythe test, respectively. Data expressed as mean±SD and the minimum level of acceptable significance was set at $p<0.05$. Different lowercase letters were used to indicate significant differences between groups.

3. Results

3.1 Al accumulation in the brain

Over time, the Al concentration in the brain of treated fish increased exponentially and peaked at 15 days and then it decreased in the organisms exposed to 20 days of treatment (Fig. 1). After 10 days of treatment, its concentration was 0.038±0.006, $p<0.05$ compared to that of the controls (0.018±0.005), for which the presence of Al in traces was in any case highlighted. After 15 days the amount of Al recovered was approximately 4-fold greater (0.071±0.013, $p<0.001$) than in the controls. Instead, after 20 days the amount of Al tended to decrease (0.030±0.015) compared to the shorter exposure times, with values closer to those found in the controls.

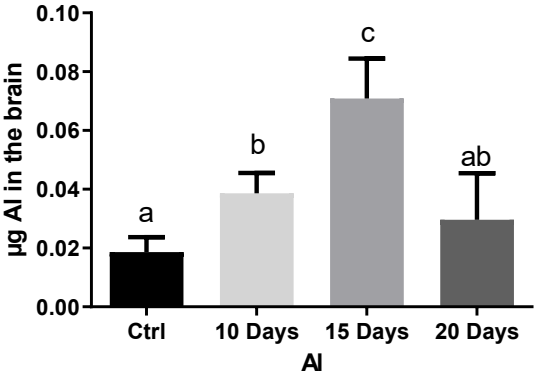

Fig. 1 Aluminium concentration in the brain of zebrafish during treatment, determined by ICP-MS.

3.2 *Morphology of brain*

3.2.1 Haematoxylin-Eosin

The general histology of zebrafish brain in control specimens showed a homogeneous tissue with well-defined structures in all the areas of the organ (Fig. 2A). The tissue appeared to be altered for all organisms exposed to Al, albeit with varying severity. Brains of organisms exposed at 10 days (Fig. 2B) and those exposed at 15 days (Fig. 2C) showed a marked cellular disorganization and a diffuse increase of edemas. Instead, the brains of the organisms exposed at 20 days to treatment (Fig. 2D) showed less edematous and disorganised tissue compared to the other treatment periods, with a parenchymal structure nearer to that of the controls.

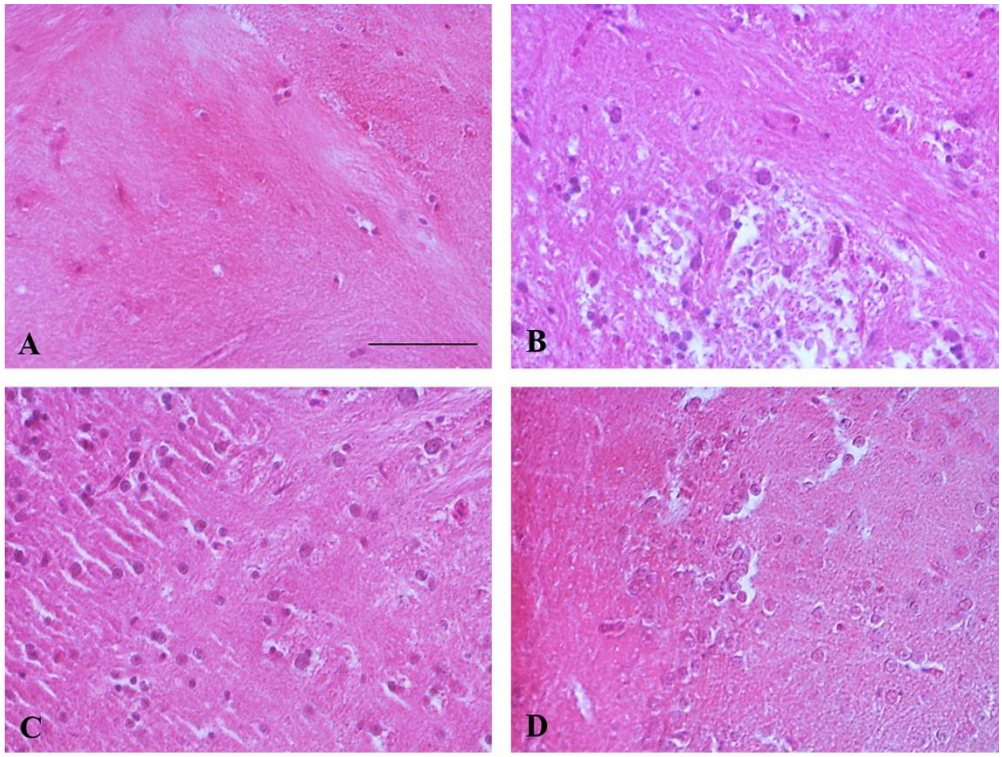

Fig. 2 Haematoxylin-Eosin staining. **A:** Brain parenchyma of control brain with normal general histology of the tissue. **B:** Brain parenchyma of brain after 10 days from treatment, the tissue appeared altered and edematous. **C:** Parenchyma of brain after 15 days, tissue still disorganised and with edemas. **D:** Parenchyma of organisms exposed to Al for 20 days, with a little altered parenchyma and more similar to that of controls. Scale bar: 50 μm.

3.2.2 Methasol fast blue

Al exposure induced alteration of myelin in the first periods of exposure. In particular, the effect was evident after 10 days of exposure, when the brains showed a decrease in MFB staining intensity in several areas evaluated, precisely, at the level of the optic tectum (Fig. 3B), telencephalon (Fig. 3F) and cerebellum/medulla oblongata (Fig. 3J) respect to the same areas evaluated in the control specimens. The latter, in fact, showed demonstrated excellent tissue contrast and clear delineation of anatomical structures, optic tectum (Fig. 3A), telencephalon (Fig. 3E) and cerebellum/medulla oblongata (Fig. 3 I). The decrease in signal intensity is due to a decrease in the affinity of the fibres for the dye and is linked to an ongoing demyelination process. Instead, after longer exposure periods 15 (Fig. 3C, G, K) and 20 (Fig. 3D, H, L) days of treatment, the fibres appeared more dye, with an increase in tissue contrast compared to the previous period of exposure, sign of tissue' increased myelination.

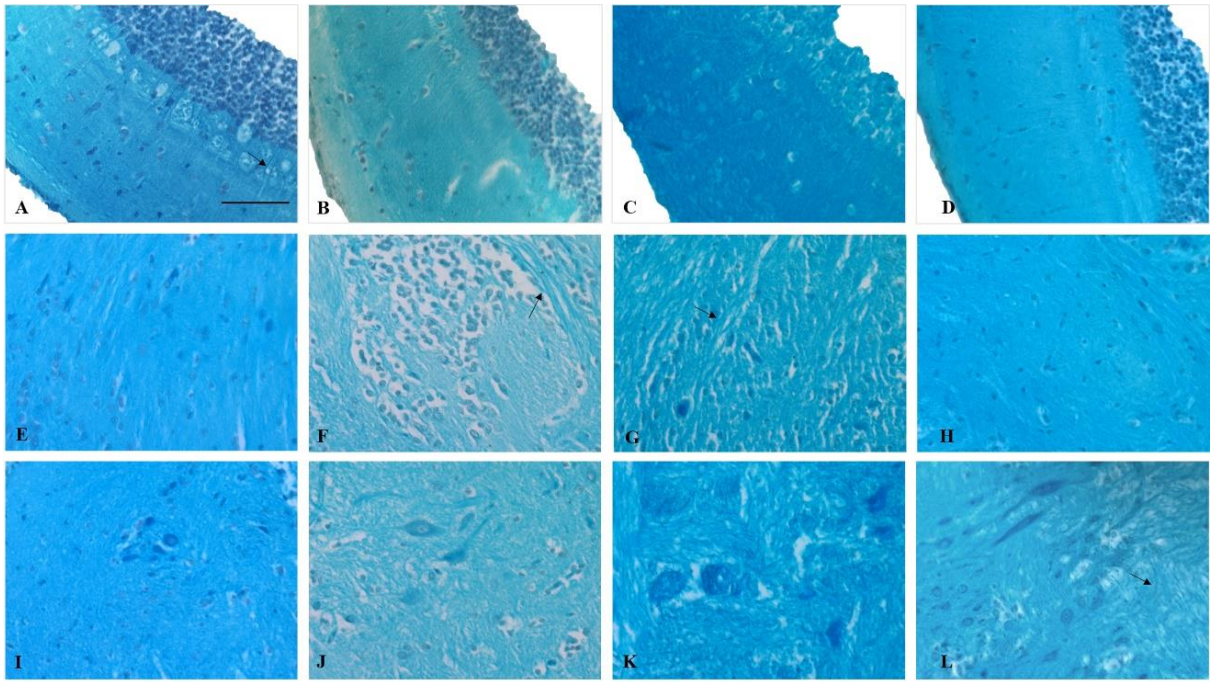

Fig. 3 Methasol Fast Blue (MFB) staining**. A-D** Optic tectum**; E-H** Telencephalon; **I-H** Cerebellum/Medulla oblongata. **A, E, I** Control. **B, F, J** Organisms' brain exposed to Al for 10 days with evident demyelination process. **C, G, K** Organisms' brain exposed to Al for 15 days. **D, H, L** Organisms' brain exposed to Al for 20 days. Arrows indicate coloured fibres. Scale bar: 50 μm.

3.2.3 Fluoro-Jade B

Application of the histology staining Fluoro-Jade B (FJ) allowed to detect possible degenerative phenomena by the identification of different fluorescent signals in control and Al-treated fish. Fluorescence was found both at the level of cell bodies and nervous processes. Moreover, the morphology of positive cells appeared variable and probably relative to different cellular types, both glial cells and neurons (Fig. 4, panel **I**). Few fluorescent signals were revealed in control brains (Fig. 4, panel **I**, A, E, I). Instead, an increase in fluorescent spots was evident in all the brains of the treated organisms. For organisms exposed to 10 (Fig. 4, panel **I**, B, F, J) and 15 days (Fig. 4, panel **I**, C, G, K) of treatment while after 20 days (Fig. 4, panel **I**) a minor presence of spots appeared in all three areas examined, optic tectum (Fig. 4, panel **I**, D), telencephalon (Fig. 4, panel **I**, H) and cerebellum/medulla oblongata (Fig. 4, panel **I**, L). Above all, the diffusion of FJ (+) cells was particular in organisms exposed to 15 days of treatment, since appeared localized in specific areas rather than homogeneously spread throughout the evaluation area (Fig. 4, panel **III**). Indeed, from the quantitative evaluation carried out, as reported in the graph (Fig. 4, panel **II**), there was an approximately 5-fold increase in spots in the 0.1 mm^2 area of the optic tectum in organisms exposed for 10 days to treatment ($p<0.05$), and further doubled ($p<0.01$) for Al-treated organisms for 15 days ($p<0.05$) than the number of fluorescent cells found in the controls. However, after 20 days of treatments this trend was reversed and the number of FJ (+) cells decreased, remaining however above the controls ($p<0.05$). In the same way also at the telencephalic level, the number of FJ (+) cells increased after 10 days ($p<0.01$) and 15 days ($p<0.001$) compared to controls and tended to decrease after 20 days ($p<0.01$). Finally, the area relating to the cerebellum and medulla oblongata appeared to be the area with more positivity than others, with an increase of about 12 ($p<0.01$) and 15 -fold ($p<0.001$) for organisms exposed to 10 and 15 days of treatment, respectively, compared to controls. Again, a slight decrease was recorded after 20 days of treatment ($p<0.01$).

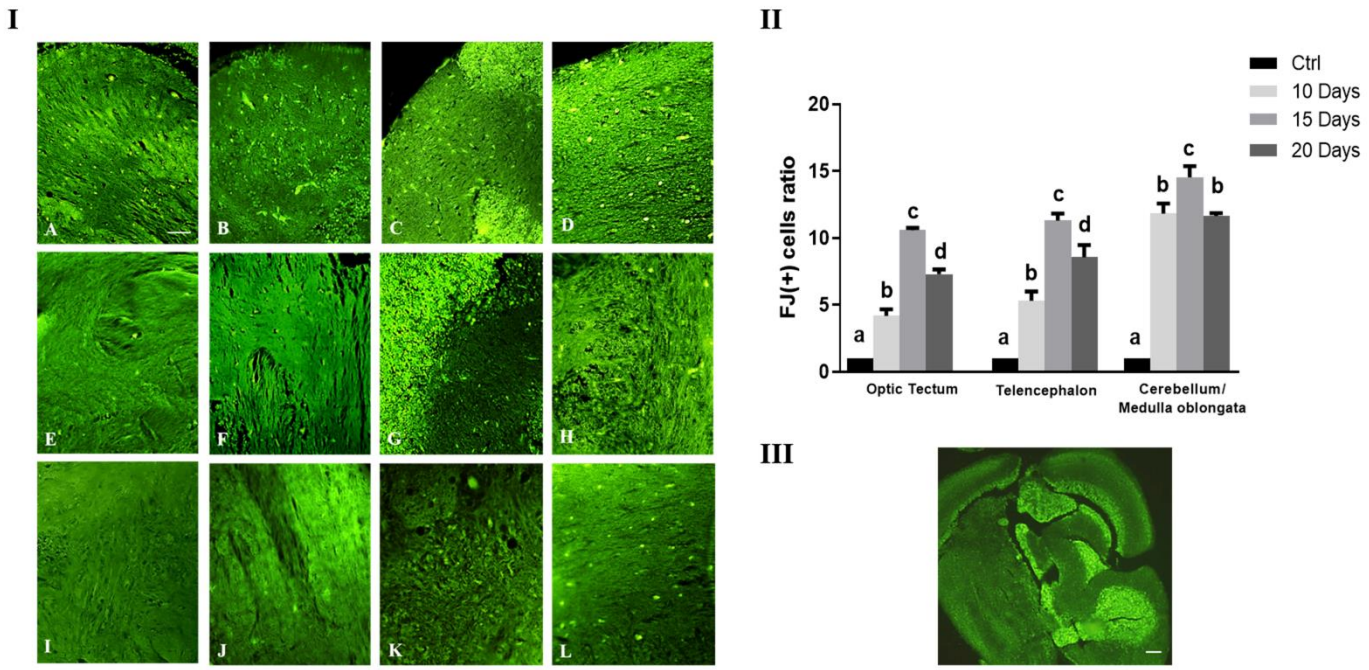

Fig. 4 Fluoro-Jade B staining. ***Panel I.*** **A-D** Optic tectum; **E-H** Telencephalon; **I-L** Cerebellum/medulla oblongata. **A, E, I** Control (Ctrl). **B, F, J** Organisms exposed to Al for 10 days**. C, G, K** Organisms exposed to Al for 15 days. **D, H, L** Organisms exposed to Al for 20 days. ***Panel II.*** Graphical representation of FJ (+) cells ratio/0.1 mm^2 in the area of the optic tectum, telencephalon, and cerebellum/medulla oblongata in adults after 10, 15 and 20 days of Al-treatment. ***Panel III***. FJ (+) cells in the section of brain of organism exposed to Al for 15 days. Scale bars: 50 µm.

3.2.4 Immunohistochemistry

From the immunohistochemistry revelation of the GFAP protein, a high presence of it emerged in the control organisms widely diffused in all the areas evaluated (Fig. 5): telencephalon (Fig. 5A), cerebellum (Fig. 5E) and medulla oblongata (Fig. 5I). No positivity in star-shaped cells (Astrocyte cells) was observed in the control samples or in those treated and it is confirmed the radial character of the glial component of the teleost brain. In the brains of fish exposed to 10 days of treatment, a decrease in positivity to the reaction was evident, in the telencephalon (Fig. 5B), cerebellum (Fig 5F) and medulla oblongata (Fig. 5J) For the organisms exposed to 15 days, on the other hand, the positivity increased and was widespread in all the areas evaluated (Fig. 5C,G,K) with a particular diffusion in the medulla oblongata (Fig. 5K). Finally, also in the organisms exposed for 20 days to Al, the positivity was uniformly distributed with glial cells that irradiated in all areas (Fig. 5D, H, L).

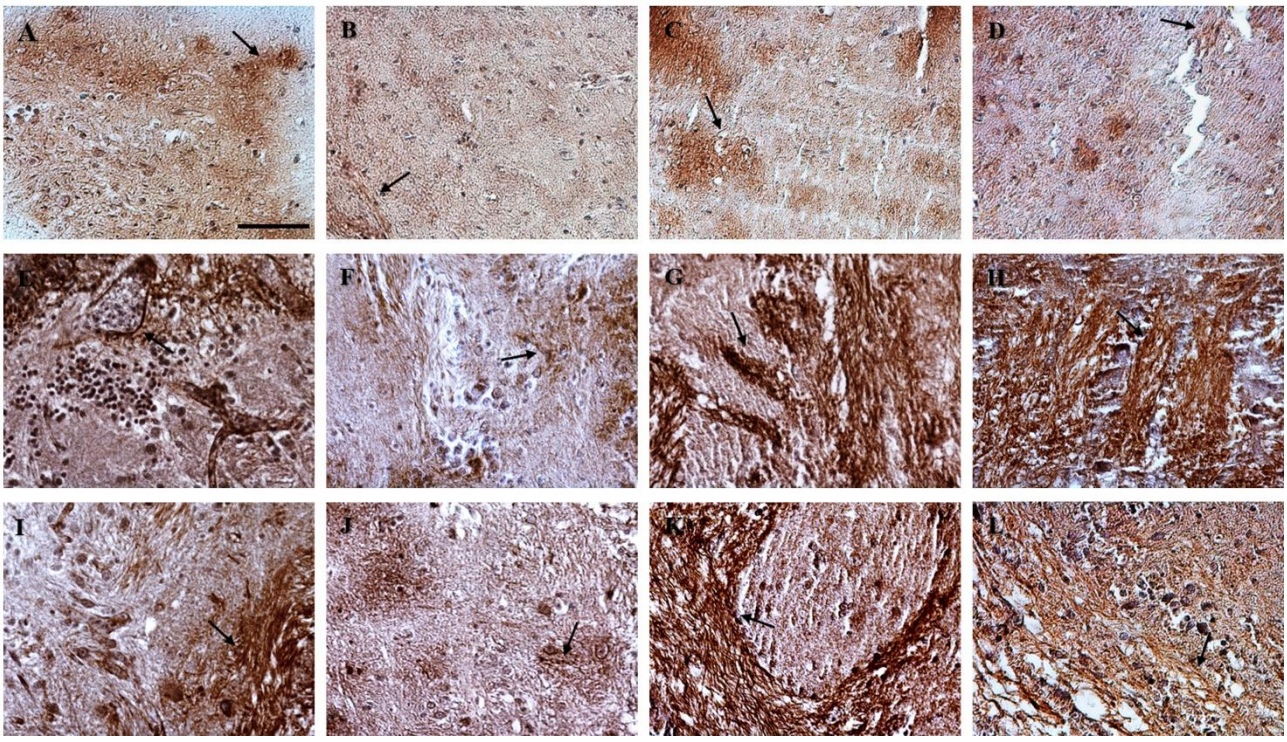

Fig. 5 Immunohistochemical detection of GFAP in zebrafish brain. **A-D** Telencephalon; **E-H**; Cerebellum **I-L** Medulla oblongata. **A, E, I** Control (Ctrl). **B, F, J** Organisms exposed to Al for 10 days**. C, G, K** Organisms exposed to Al for 15 days. **D, H, L** Organisms exposed to Al for 20 days. The arrows indicate coloured fibres. Scale bar: 50 µm.

3.3 *Gene expression*

Based on RT-qPCR analyses, the expression levels of the gene group showed a significant association of Parkinsonism in zebrafish exposed to Al (Fig. 6). Indeed, all genes evaluated were up-regulated in treated organisms compared to controls, albeit with different trends. The *SYNB, SYNB2, SNCGB* genes showed the same trend in their expression, increased significantly for Al-treated for 10 days ($p<0.01$), decreased for organisms exposed at 15 days, coming to be similar to those of controls and increased again after 20 days exposure ($p<0.001$). Even the *SNCGA* and *PARK7* genes had a similar trend with a significant increase in expression after 10 days ($p<0.01$), a slight decrease after 15 days ($p<0.01$) and a renewed increase after 20 days of treatment ($p<0.001$ for *SNCGA* and $p<0.01$ for *PARK7*). On the other hand, no change was recorded in the expression of the *PARK2* gene after 10 days of treatment compared to controls, its levels become significantly higher only after 15 and 20

days of exposure (p<0.05). A linear dose-dependent increase was instead recorded for the expression of the *SEPT3, PINK1* gene with a significant increase after 10 days of treatment compared to controls (p<0.05 for *SEPT3* expression and p<0.001 for *PINK1* expression), which increased further after 15 days of Al exposure (p<0.01) and again after 20 days (p<0.01 for *SEPT3* expression and p<0.001 for *PINK1* expression). The expression of *CHCHD2* increased in organisms exposed to 10-15 days compared to controls (p<0.01) and remained constant between them, but a further and high increase was evident after 20 days of exposure (p<0.001). Finally, *GIGYF2* was the only gene whose expression, while increasing for organisms exposed at 10 and 15 days (p<0.05), decreased in Al-treated after 20 days up to the physiological levels expressed in the controls.

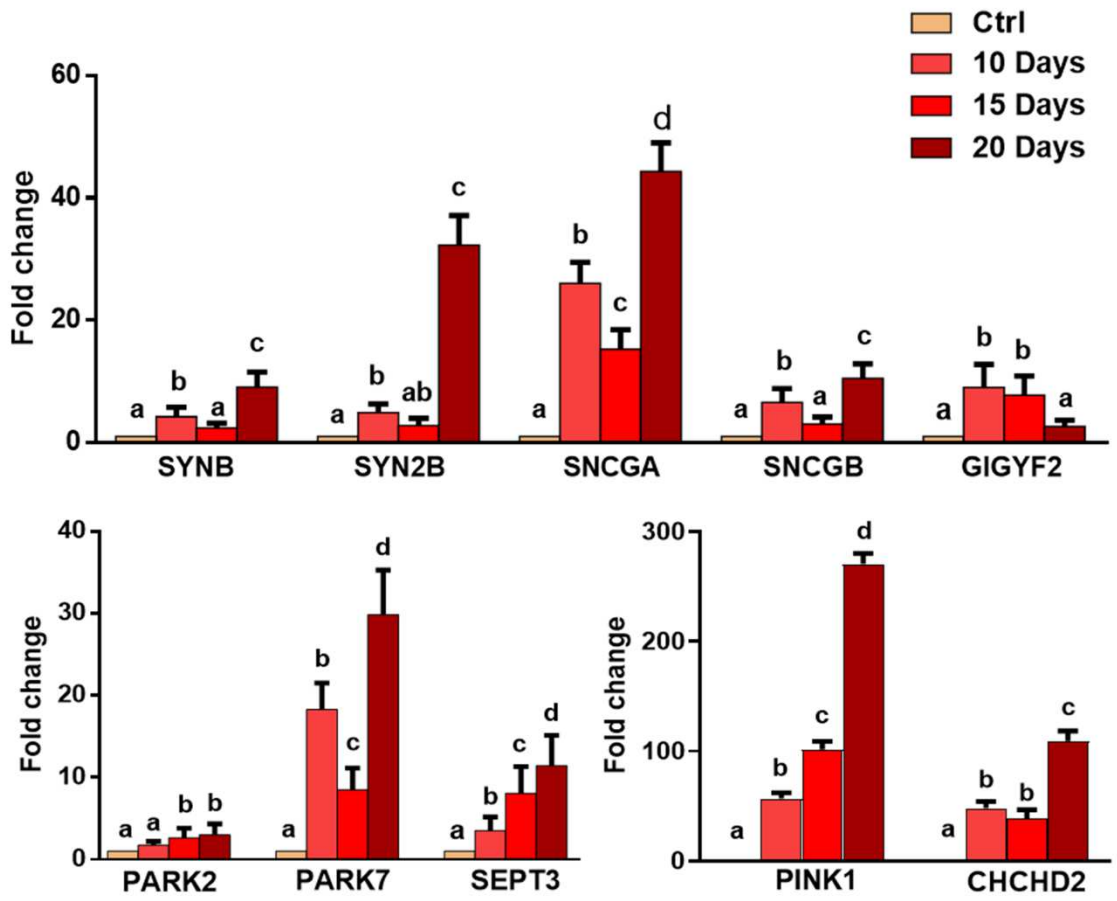

Fig. 6 Expression pattern comparison by means of RT-qPCR. Gene expression levels were normalized to the reference transcript *β-ACTIN* and calculated by the $2^{-\Delta\Delta Ct}$ method.

4. Discussion

The aim of this work was to evaluate the neurotoxic effect of Al and its role in inducing neurodegeneration.

In our previous studies, we have already demonstrated the toxic effect of Al both on zebrafish in the early stages of development, in which the metal induced alteration of motility (Capriello et al., 2019) and variation of the glial marker GFAP expression (Monaco et al., 2017b) and in the adult phase, in which Al induced alteration of swimming ability and behaviour but also alteration of the oxidative state of the brain (Capriello et al., 2021). The latter considered the mechanism of action underlying neurotoxicity (Igbokwe et al., 2019; Jang and Surh, 2002). In this regard, the effects were greater after low doses (Capriello et al., 2019) or short exposure (Capriello et al., 2021) and improving at higher concentrations, or long Al-exposure (Capriello et al., 2019; 2021). In this light, the quantity of Al penetrated into the brain after 10, 15 and 20 days were evaluated and the analysis showed a peak of its accumulation after 15 days of treatment and a lower presence of Al after 20 days of exposure. It is known that Al can rapidly enter the brain, and accumulate at the level of extracellular and the cerebrospinal fluids (Krewski et al., 2007). In this case, the metal accumulation that we detected is clearly linked to the alteration of the behavioural response recorded at 15 days of treatment (Capriello et al., 2021), which coincides with the maximum accumulation recorded. Even the decrease in the concentration of Al after 20 days is in agreement with the trend of the improvement of the activities assessed on the same day (Capriello et al., 2021). Moreover, this Al accumulation is agreed with the increased amount of Al found in humans' brains affected by neurodegenerative diseases (Ahmed and Santosh, 2010; Mirza et al., 2017; Zatta et al., 2003). It is also necessary to specify that Al concentration, albeit low, it is found also in the controls' brain. It is a normal condition, already found on the occasion of the evaluation of other metals such as cadmium (Cd) (Favorito et al., 2011), as these metals are also normally present in water for human consumption (Igbokwe et al., 2019; Järup et al., 1998) and used for housing of fish, albeit with very low concentrations, and can therefore

penetrate the organ and accumulate over time. Even the structure of the brain' parenchyma was particularly altered in the first periods of exposure and stabilized after 20 days of treatment. The alterations found such as the edematous alterations, they have already observed in brain of Al-exposed rats (Singla and Dhawan, 2014), but also in lizard (Favorito et al., 2017) and zebrafish (Monaco et al., 2016) exposed to Cd, another metal considered neurotoxic. These alterations are probably caused by alteration of brains blood vessels and related to the damages on the integrity of blood-brain barrier (BBB), the main cause of formation of edemas (Scorticati et al., 2004). The alterations of the tissue were also visible with the Methasol fast blue staining, which was used to verify the possible effect of Al on myelin. In this regard, a decrease in staining affinity occurred only in organisms exposed to 10 days of treatment, and a resumption of chromatic impact was recorded in subsequent exposure periods. This alteration of the tincture is associated with a structural and chemical alteration of the myelin (Goldmann et al., 2013; Way et al., 2015) which can occur in connection with tissue degeneration, as shown in Cuprizone-exposed mice (Khodanovich et al., 2017) or also in zebrafish exposed to Cd (Monaco et al., 2016).

Furthermore, a quick recovery after time such as that occurred after 15 and 20 days found confirmation in the literature, where it is indicated that there is a varied course of demyelinating diseases, for which in some cases, the examples of demyelinating appeared like single manifestations, one or two exacerbations during the entire course of the disorder (Allen et al., 1998). Probably, as hypothesized by Allen et al., (1998) the recovery occurred due to improvement in membrane electrical functions and/or repair of myelin.

The neurotoxic role of Al has been repeatedly supposed in relation to an increase in oxidative stress, induced inflammation or neurodegenerative processes (Bondy et al., 2014; Pratico et al., 2002). In this case, it was evident that Al induced the activation of neurogenerative processes in the different areas evaluated by Fluoro-Jade B staining. The use of this dye allowed evidence degeneration phenomena both for neurons and glial cells (Ehara and Ueda, 2009; Damjanac et al., 2007) considering different types of cell death such as apoptosis and necrosis. (Zhou et al., 2005). An

increase in this effect finds similarity in zebrafish brains exposed to the Cd (Monaco et al., 2017a). Moreover, it is also known that Al can have a pro-apoptotic activity (Lukiw et al., 2005) and this certainly contributed to a greater presence of FJ (+) cells found after 10 and 15 days of exposure. The decrease in neurodegeration occurring after 20 days of treatment is in agreement with the previous data obtained (Capriello et al., 2021), probably due to the activation of an efficient adaptive mechanism as supposed in zebrafish after exposure to different pollutants (Acosta et al., 2016; Avallone et al., 2015; Pilehvar et al., 2020). Moreover, this condition is reflected in the improvement of the oxidative state conditions and activation of the antioxidant system evaluated previously (Capriello et al., 2021). Instead, the particular localization of FJ (+) cells for organisms exposed to 15 days of treatment could be linked to the susceptibility to degeneration of specific groups of cells, such as dopaminergic neurons, are more susceptible to oxidative stress and environmental stressors (Dias et al., 2013). This phenomenon could also justify the altered, anxiogenic and asocial behaviour (Capriello et al., 2021), recorded at this time (15days), given the high correlation between behavioural changes and neurodegeneration induced in zebrafish (Tierney, 2011). However, further studies are needed to confirm this deduction. From the immunohistochemical detection of the GFAP protein it is evident that Al-treatment induced a reduction in expression after 10 days of treatment, in accordance with the data relating to tissue demyelization. Indeed, as reported by Middeldorp and Hol, (2011), GFAP is involved in the maintenance of normal myelination of the nervous system, so the simultaneous decrease of both is reasonable. Moreover, glial cells are essential for the formation and maintenance of the BBB (Alvarez et al., 2013) and the alterations of these cells and their extensions may, therefore, alter the integrity of these barriers, leading to an alteration in the brain parenchyma and the formation of edemas, in this case, more present in the brain of organisms exposed to Al for 10 days. Furthermore, a reduction in GFAP expression is often linked to a neurotoxic effect induced by polluted, as previously reported in the zebrafish and lizard brain, in which reduction of GFAP expression was observed after Cd exposure (Favorito et al., 2017; Monaco et al., 2016) but also verified in cell cultures of rat brain exposed to heavy metal' mixture (Rai et al., 2013). An

increase in GFAP expression was recorded in longer exposure times and this phenomenon could be linked to the increase in the activity of antioxidant enzymes previously evaluated (Capriello et al., 2021), according to the data reported by Singla and Dhawan, (2017) in rats exposed to high concentrations of Al. Furthermore, this effect can also be correlated with the evaluation of the expression of the markers involved in Parkinsonism which are increasing after 15 and 20 days of treatment. Indeed, as reported by several studies in relation to PD, GFAP expression did not decrease, but it was found that it was not altered (Abdo et al., 2004; Banati et al., 1998; Holmberg et al., 1998) or sometimes even an increase in the density of GFAP positive cells has been recorded (Damier et al., 1993; Thannickal et al., 2007).

PD is an oxidative stress-induced neurodegenerative disease that implies a gradual loss of dopaminergic neurons in the substantia nigra and α-synuclein agglutination (Gandhi and Abramov, 2012; Pisoschi and Pop, 2015). In the light of the results, that emerged in the previous study regarding the oxidative state of the brain (Capriello et al., 2021), marker genes related to parkinsonism were evaluated and it emerged that the expression of all the genes evaluated was altered by Al-treatment.

The genes coding for the protein α-synuclein and synapsin, which play a fundamental role in the evolution of PD and in the formation of Lewy bodies (Longhena et al., 2018) were up-regulated especially after 10 and 20 days of Al-exposure; while the expression of the *SEPT3* gene, encoding for the Septin 3 protein, increased with increasing exposure period.

It is known that the increased expression of these proteins is linked to the development of PD (Faustini et al., 2020; Marttinen et al., 2015) and that Al can promote the aggregation of α-synuclein, further enhancing the process of neurodegeneration (Milanese et al., 2001; Singla and Dhawan, 2017; Uversky et al., 2001). Instead, it is not known the link between exposure to Al and the expression of synapsin and septin 3. However, for the genes linked to the expression of α-synuclein and synapsin, there was a decrease in their expression on the 15th day of exposure in conjunction with the increase in the activity of antioxidant enzymes (Capriello et al., 2021) and with the increase in the expression of genes related to PD, but whose overexpression assumes a protective connotation such as the

PARK2 gene, which codes for the Parkin protein (Darios et al., 2003), *PINK1* (Haque et al., 2008) and CHCHD2 gene (Imai et al. 2019) . It is important to underline also that after 20 days of Al-exposure the *PINK1* expression was further increased and reaches to be about 270-fold higher than that recorded in the controls' brain; this could be linked to the decrease in neurodegeneration recorded at this time of exposure, given the known anti-apoptotic role of this protein (Petit et al., 2005). Even the expression of the PARK7 gene, coding for the DJ-1 protein, was increased after 20 days of treatment, probably in response to the altered conditions of oxidative stress (Lev et al., 2008), as it is known that its overexpression has a neuroprotective role, linked to its antioxidant action (Canet-Aviles et al., 2004; Edson et al., 2019). Finally, the expression of the *GIGYF2* gene, coding for the Park11 protein, showed a different trend: it increased for the organisms exposed to 10 and 15 days of treatment and decreased for the organisms exposed to 20 days as if to return to the physiological levels shown by the controls. It is known that over-expression of *GIGYF2* appears to alter receptor trafficking and can may regulate 'fast' membrane recycling (Higashi et al., 2010). For this reason, its over-expression after 10 and 15 days of treatment could be due to the need to support the increase in locomotor activity it found in the first periods of exposure (Capriello et al., 2021). Its subsequent reduction could also in this case be linked to an improvement in the general conditions recorded at the maximum exposure time.

Overall, the gene expression data does not provide a complete picture of the link between Al-exposure and the onset of PD, which needs further investigation, however, supports and confirms the ability of Al to have a neurotoxic action and a role of neurodegeneration activation.

5. Conclusion

All the information presented in this study helped to elucidate the possible mechanism of Al- induced neurotoxicity. Having analysed the effects from different points of view it emerged that this metal can alter the histology of tissue, cause neurodegeneration and induce demyelination especially in the first periods of exposure. This trend was also followed by the expression of GFAP. At longer exposure

times there was an improvement/stabilization of the overall neurological conditions. This effect was also supported by genetic expression data which, in addition to showing an altered expression of genes closely linked to parkinsonism, also shows an increased expression of genes with a neuroprotective effect.

Conflict of interest

The authors declare that they have no financial interests or personal relationships that could influence the content of the paper.

References

Abdo, W.F., De Jong, D., Hendriks, J.C., Horstink, M.W., Kremer, B.P., Bloem, B.R., Verbeek, M.M., 20004. Cerebrospinal fluid analysis differentiates multiple system atrophy from Parkinson's disease. Mov Disord.; 19(5):571-9. DOI: 10.1002/mds.10714.

Acosta, D.D.S., Danielle, N.M., Altenhofen, S., Luzardo, M.D., Costa, P.G., Bianchini, A., Bonan, C.D., da Silva, R.S., Dafre, A.L., 2016. Copper at low levels impairs memory of adult zebrafish (Danio rerio) and affects swimming performance of larvae. Comp. Biochem. Physiol. C Toxicol. Pharmacol.; 185-186:122-130. DOI: 10.1016/j.cbpc.2016.03.008.

Agency for Toxic Substances and Disease (ATSDR), 2008. Toxicological Profile for Aluminum. CAS: 7429-90-5.

Ahmed, S.S., Santosh, W., 2010. Metallomic profiling and linkage map analysis of early Parkinson's disease: a new insight to aluminum marker for the possible diagnosis. PLoS One.; 5(6):e11252. DOI: 10.1371/journal.pone.0011252.

Alfrey, A.C., Solomons, C.C., 1976. Bone pyrophosphate in uremia and its association with extraosseous calcification. J. Clin. Invest.; 57(3):700-5. DOI: 10.1172/JCI108327.

Allen D.N., Sprenkel D.G., Heyman R.A., Schramke C.J., Heffron N.E., 1998. Evaluation of Demyelinating and Degenerative Disorders. In: Goldstein G., Nussbaum P.D., Beers S.R. (eds) Neuropsychology. Human Brain Function (Assessment and Rehabilitation). Springer, Boston, MA. https://doi.org/10.1007/978-1-4899-1950-2_9.

Alvarez, J.I., Katayama, T., Prat, A., 2013. Glial influence on the blood brain barrier. Glia; 61(12):1939-58. doi: 10.1002/glia.22575.

Arain, M.S., Afridi, H.I., Kazi, T.G., Talpur, F.N., Arain, M.B., Kazi, A., Arain, S.A., Ali, J., 2015. Correlation of aluminum and manganese concentration in scalp hair samples of patients having neurological disorders. Environ. Monit. Assess.; 187(2):10. DOI: 10.1007/s10661-014-4172-0.

Avallone, B., Agnisola, C., Cerciello, R., Panzuto, R., Simoniello, P., Cretì, P., Motta, C.M., 2015. Structural and functional changes in the zebrafish (Danio rerio) skeletal muscle after cadmium exposure. Cell Biol. Toxicol.; 31(6):273-83. DOI: 10.1007/s10565-015-9310-0.

Babin, P.J., Goizet, C., Raldúa, D., 2014. Zebrafish models of human motor neuron diseases: advantages and limitations. Prog Neurobiol.; 118:36-58. DOI: 10.1016/j.pneurobio.2014.03.001.

Bambino, K., Chu, J., 2017. Zebrafish in Toxicology and Environmental Health. Curr. Top Dev. Biol.; 124:331-367. DOI: 10.1016/bs.ctdb.2016.10.007.

Banati, R.B., Daniel, S.E., Blunt, S.B., 1998. Glial pathology but absence of apoptotic nigral neurons in long-standing Parkinson's disease. Movement Disorders : Official Journal of the Movement Disorder Society.; 13(2):221-227. DOI: 10.1002/mds.870130205.

Becaria, A., Campbell, A., Bondy, S.C., 2002. Aluminum as a toxicant. Toxicol Ind Health.; 18(7):309-20. DOI: 10.1191/0748233702th157oa.

Bjorklund, G., Stejskal, V., Urbina, M.A., Dadar, M., Chirumbolo, S., Mutter, J., 2018. Metals and Parkinson's Disease: Mechanisms and Biochemical Processes. Curr Med Chem.; 25(19):2198-2214. DOI: 10.2174/0929867325666171129124616.

Bondy, S.C., 2014. Prolonged exposure to low levels of aluminum leads to changes associated with brain aging and neurodegeneration. Toxicology.; 315:1-7. DOI: 10.1016/j.tox.2013.10.008..

Bondy, S.C., 2016. Low levels of aluminum can lead to behavioral and morphological changes associated with Alzheimer's disease and age-related neurodegeneration. Neurotoxicology; 52:222-9. DOI: 10.1016/j.neuro.2015.12.002.

Canet-Avilés, R.M., Wilson, M.A., Miller, D.W., Ahmad, R., McLendon, C., Bandyopadhyay, S., Baptista, M.J., Ringe, D., Petsko, G.A., Cookson, M.R., 2004. The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. Proc. Natl. Acad. Sci U S A.; 101(24):9103-8. DOI: 10.1073/pnas.0402959101.

Capriello, T., Félix, L.M., Monteiro, S.M., Santos, D., Cofone, R., Ferrandino, I., 2021. Exposure to aluminium causes behavioural alterations and oxidative stress in the brain of adult zebrafish. Environ. Toxicol. Pharmacol.; 85:103636. DOI: 10.1016/j.etap.2021.103636.

Capriello, T., Grimaldi, M.C., Cofone, R., D'Aniello, S., Ferrandino, I., 2019. Effects of aluminium and cadmium on hatching and swimming ability in developing zebrafish. Chemosphere; 222:243-249. DOI: 10.1016/j.chemosphere.2019.01.140.

Colomina, M.T., Peris-Sampedro, F., 2017. Aluminum and Alzheimer's Disease. Adv Neurobiol. 2017;18:183-197. DOI: 10.1007/978-3-319-60189-2_9.

Damier, P., Hirsch, E.C., Zhang, P., Agid, Y., Javoy-Agid, F., 1993. Glutathione peroxidase, glial cells and Parkinson's disease. Neuroscience.; 52(1):1-6. DOI: 10.1016/0306-4522(93)90175-f.

Damjanac, M., Rioux Bilan, A., Barrier, L., Pontcharraud, R., Anne, C., Hugon, J., Page, G., 2007. Fluoro-Jade B staining as useful tool to identify activated microglia and astrocytes in a mouse transgenic model of Alzheimer's disease. Brain Res; 1128(1):40-9. DOI: 10.1016/j.brainres.2006.05.050.

Darios, F., Corti, O., Lücking, C.B., Hampe, C., Muriel, M.P., Abbas, N., Gu, W.J., Hirsch, E.C., Rooney, T., Ruberg, M., Brice, A., 2003. Parkin prevents mitochondrial swelling and cytochrome c release in mitochondria-dependent cell death. Hum. Mol. Genet.; 12(5):517-26. DOI: 10.1093/hmg/ddg044.

Dias, V., Junn, E., Mouradian, M.M., 2013. The role of oxidative stress in Parkinson's disease. J Parkinsons Dis.; 3(4):461-91. DOI: 10.3233/JPD-130230.

Edson, A.J., Hushagen, H.A., Frøyset, A.K., Elda, I., Khan, E.A., Di Stefano, A., Fladmark, K.E., 2019. Dysregulation in the Brain Protein Profile of Zebrafish Lacking the Parkinson's Disease-Related Protein DJ-1. Mol Neurobiol.; 56(12):8306-8322. DOI: 10.1007/s12035-019-01667-w.

Ehara, A., Ueda, S., 2009. Application of Fluoro-Jade C in acute and chronic neurodegeneration models: utilities and staining differences. Acta Histochem. Cytochem.; 42(6):171-9. DOI: 10.1267/ahc.09018.

Exley, C., 2006. Aluminium and iron, but neither copper nor zinc, are key to the precipitation of beta-sheets of Abeta42 in senile plaque cores in Alzheimer's disease. J. Alzheimers Dis.; 10(2-3):173-7. DOI: 10.3233/jad-2006-102-305.

Exley, C., House, E.R., 2011. Aluminium in the human brain. Monatsh Chem. 142(4):357-63. https://doi.org/10.1007/s00706-010-0417-y.

Faustini, G., Longhena, F., Bruno, A., Bono, F., Grigoletto, J., La Via, L., Barbon, A., Casiraghi, A., Straniero, V., Valoti, E., Costantino, G., Benfenati, F., Missale, C., Pizzi, M., Spillantini, M.G., Bellucci, A., 2020. Alpha-synuclein/synapsin III pathological interplay boosts the motor response to methylphenidate. Neurobiol. Dis.; 138:104789. DOI: 10.1016/j.nbd.2020.104789.

Favorito, R., Chiarelli, G., Grimaldi, M.C., De Bonis, S., Lancieri, M., Ferrandino, I., 2011. Bioaccumulation of cadmium and its cytotoxic effect on zebrafish brain. Chem. Ecol.; 27(2),39-46. https://doi.org/10.1080/02757540.2011.625937.

Favorito, R., Monaco, A., Grimaldi, M.C., Ferrandino, I., 2017. Effects of cadmium on the glial architecture in lizard brain. Eur. J. Histochem.; 61,2734. DOI: 10.4081/ejh.2017.2734.

Gandhi, S., Abramov, A.Y., 2012. Mechanism of oxidative stress in neurodegeneration. Oxid. Med. Cell. Longev. Volume 2012, Article ID 428010:11.DOI:10.1155/2012/428010.

Goldmann, T., Wieghofer, P., Müller, P.F., Wolf, Y., Varol, D., Yona, S., Brendecke, S.M., Kierdorf, K., Staszewski, O., Datta, M. and Luedde, T., 2013. A new type of microglia gene targeting shows TAK1 to be pivotal in CNS autoimmune inflammation. Nature neuroscience, 16(11); 1618:1626. https://doi.org/10.1038/nn.3531.

Haque, M.E., Thomas, K.J., D'Souza, C., Callaghan, S., Kitada, T., Slack, R.S., Fraser, P., Cookson, M.R., Tandon, A., Park, D.S., 2008. Cytoplasmic Pink1 activity protects neurons from dopaminergic neurotoxin MPTP. Proc. Natl. Acad. Sci U S A.; 105(5):1716-21. DOI: 10.1073/pnas.0705363105.

Higashi, S., Iseki, E., Minegishi, M., Togo, T., Kabuta, T., Wada, K., 2010. GIGYF2 is present in endosomal compartments in the mammalian brains and enhances IGF-1-induced ERK1/2 activation. J Neurochem.; 115(2):423-37. DOI: 10.1111/j.1471-4159.2010.06930.x.

Holmberg, B., Rosengren, L., Karlsson, J.E., Johnels, B., 1998. Increased cerebrospinal fluid levels of neurofilament protein in progressive supranuclear palsy and multiple-system atrophy compared with Parkinson's disease. Mov Disord.; 13(1):70-7. DOI: 10.1002/mds.870130116.

Igbokwe, I.O., Igwenagu, E., Igbokwe, N.A., 2019. Aluminium toxicosis: a review of toxic actions and effects. Interdiscip Toxicol.; 12(2):45-70. DOI: 10.2478/intox-2019-0007.

Imai, Y., Meng, H., Shiba-Fukushima, K., Hattori, N., 2019. Twin CHCH Proteins, CHCHD2, and CHCHD10: Key Molecules of Parkinson's Disease, Amyotrophic Lateral Sclerosis, and Frontotemporal Dementia. Int J Mol Sci.; 20(4):908. DOI: 10.3390/ijms20040908.

Jang, J.H., Surh, Y.J., 2002. beta-Amyloid induces oxidative DNA damage and cell death through activation of c-Jun N terminal kinase. Ann. N. Y. Acad. Sci.; 973:228-36. DOI: 10.1111/j.1749-6632.2002.tb04639.x.

Järup, L., Berglund, M., Elinder, C.G., Nordberg, G., Vahter, M., 1998. Health effects of cadmium exposure--a review of the literature and a risk estimate. Scand. J. Work Environ. Health.; 24 Suppl 1:1-51. Erratum in: Scand J Work Environ Health;24(3):240.

Jones, K., Linhart, C., Hawkins, C., Exley, C., 2017. Urinary Excretion of Aluminium and Silicon in Secondary Progressive Multiple Sclerosis. EBioMedicine.; 26:60-67. DOI: 10.1016/j.ebiom.2017.10.028.

Kawahara, M., Kato-Negishi, M., 2011. Link between Aluminum and the Pathogenesis of Alzheimer's Disease: The Integration of the Aluminum and Amyloid Cascade Hypotheses. Int. J. Alzheimers Dis.; 2011:276393. DOI: 10.4061/2011/276393.

Khodanovich, M.Y., Sorokina, I.V., Glazacheva, V.Y., Akulov, A.E., Nemirovich-Danchenko, N.M., Romashchenko, A.V., Tolstikova, T.G., Mustafina, L.R., Yarnykh, V.L., 2017. Histological validation of fast macromolecular proton fraction mapping as a quantitative myelin imaging method in the cuprizone demyelination model. Sci Rep.; 7:46686. DOI: 10.1038/srep46686.

Klein, G.L., 2019. Aluminum toxicity to bone: A multisystem effect? Osteoporos Sarcopenia;5(1):2-5. DOI: 10.1016/j.afos.2019.01.001.

Kongerud, J., Søyseth, V., 2014. Respiratory disorders in aluminum smelter workers. J. Occup. Environ. Med.; 56(5 Suppl):S60-70. DOI: 10.1097/JOM.0000000000000105.

Krewski, D., Yokel, R.A., Nieboer, E., Borchelt, D., Cohen, J., Harry, J., Kacew, S., Lindsay, J., Mahfouz, A.M., Rondeau, V., 2007. Human health risk assessment for aluminium, aluminium oxide, and aluminium hydroxide. J. Toxicol. Environ. Health B Crit. Rev.; 10 Suppl 1(Suppl 1):1-269. DOI: 10.1080/10937400701597766. Erratum in: J Toxicol Environ Health B Crit Rev.;11(2):147.

Lev, N., Ickowicz, D., Melamed, E., Offen, D., 2008. Oxidative insults induce DJ-1 upregulation and redistribution: implications for neuroprotection. Neurotoxicology; 29(3):397-405. DOI: 10.1016/j.neuro.2008.01.007.

Longhena, F., Faustini, G., Varanita, T., Zaltieri, M., Porrini, V., Tessari, I., Poliani, P.L., Missale, C., Borroni, B., Padovani, A., Bubacco, L., Pizzi, M., Spano, P., Bellucci, A., 2018. Synapsin III is a key component of α-synuclein fibrils in Lewy bodies of PD brains. Brain Pathol.; 28(6):875-888. DOI: 10.1111/bpa.12587.

Lukiw, W.J., Percy, M.E., Kruck, T.P., 2005. Nanomolar aluminum induces pro-inflammatory and pro-apoptotic gene expression in human brain cells in primary culture. J Inorg. Biochem.; 99(9):1895-8. DOI: 10.1016/j.jinorgbio.2005.04.021.

Marttinen, M., Kurkinen, K.M., Soininen, Haapasalo, A., Hiltunen, M., 2015. Synaptic dysfunction and septin protein family members in neurodegenerative diseases. Mol Neurodegeneration ;10:16. https://doi.org/10.1186/s13024-015-0013-z

Middeldorp, J., Hol, E.M., 2011. GFAP in health and disease. Prog Neurobiol.; 93(3):421-43. DOI: 10.1016/j.pneurobio.2011.01.005.

Milanese, M., Lkhayat, M.I., Zatta, P., 2001. Inhibitory effect of aluminum on dopamine beta-hydroxylase from bovine adrenal gland. J Trace Elem. Med. Biol.; 15(2-3):139-41. DOI: 10.1016/s0946-672x(01)80057-2.

Mirza, A., King, A., Troakes, C., Exley, C., 2017. Aluminium in brain tissue in familial Alzheimer's disease. J Trace Elem Med Biol.; 40:30-36. DOI: 10.1016/j.jtemb.2016.12.001.

Monaco, A., Capriello, T., Grimaldi, M.C., Schiano, V., Ferrandino I., 2017a. Neurodegeneration in zebrafish embryos and adults after cadmium exposure. Journal List. Eur. J. Histochem.; 61(4); 19PMC5656802. DOI: 10.4081/ejh.2017.2833.

Monaco, A., Grimaldi, M.C., Ferrandino, I., 2016. Neuroglial alterations in the zebrafish brain exposed to cadmium chloride. J. Appl. Toxicol.; 36(12),1629-38. DOI: 10.1002/jat.3328.

Monaco, A., Grimaldi, M.C., Ferrandino, I., 2017b. Aluminium chloride-induced toxicity in zebrafish larvae. J. Fish. Dis.; 40(5), 629-35. DOI: 10.1111/jfd.12544.

Morris, G., Puri, B.K. & Frye, R.E., 2017. The putative role of environmental aluminium in the development of chronic neuropathology in adults and children. How strong is the evidence and what could be the mechanisms involved?. Metab. Brain Dis. 32;1335:1355 . https://doi.org/10.1007/s11011-017-0077-2.

Petit, A., Kawarai, T., Paitel, E., Sanjo, N., Maj, M., Scheid, M., Chen, F., Gu, Y., Hasegawa, H., Salehi-Rad, S., Wang, L., Rogaeva, E., Fraser, P., Robinson, B., St George-Hyslop, P., Tandon, A., 2005. Wild-type PINK1 prevents basal and induced neuronal apoptosis, a protective effect abrogated by Parkinson disease-related mutations. J Biol Chem.; 280(40):34025-32. DOI: 10.1074/jbc.M505143200.

Pilehvar, A., Town, R.M., Blust, R., 2020. The effect of copper on behaviour, memory, and associative learning ability of zebrafish (Danio rerio). Ecotoxicol. Environ. Saf.; 188-109900. DOI: 10.1016/j.ecoenv.2019.109900.

Pisoschi, A.M., Pop, A., 2015. The role of antioxidants in the chemistry of oxidative stress: A review. Eur J Med Chem.; 97:55-74. DOI: 10.1016/j.ejmech.2015.04.040.

Pratico, D., Uryu, K., Sung, S., Tang, S., Trojanowski, J.Q., Lee, V.M., 2002. Aluminum modulates brain amyloidosis through oxidative stress in APP transgenic mice.FASEB J. 16; 1138:1140. doi: 10.1096/fj.02-0012fje.

Rai, A., Maurya, S.K., Sharma, R., Ali, S., 2013. Down-regulated GFAPα: a major player in heavy metal induced astrocyte damage. Toxicol. Mech. Methods.; 23(2):99-107. DOI: 10.3109/15376516.2012.721809.

Sarath Babu, N., Murthy Ch.L, Kakara, S., Sharma, R., Brahmendra, Swamy. C.V., Idris, M.M., 2016. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine induced Parkinson's disease in zebrafish. Proteomics; 16(9):1407-20. DOI: 10.1002/pmic.201500291.

Schiffer, D., Cordera, S., Cavalla, P., Migheli, A., 1996. Reactive astrogliosis of the spinal cord in amyotrophic lateral sclerosis. J. Neurol. Sci. ; 139 Suppl:27-33. DOI: 10.1016/0022-510x(96)00073-1.

Scorticati, C., Prestifilippo, J.P., Eizayaga, F.X., Castro, J.L., Romay, S., Fernández, M.A., Lemberg, A., Perazzo, J.C., 2004. Hyperammonemia, brain edema and blood-brain barrier alterations in prehepatic portal hypertensive rats and paracetamol intoxication. World J. Gastroenterol.; 10(9):1321-4. DOI: 10.3748/wjg.v10.i9.1321.

Singla, N., Dhawan, D.K., 2014. Zinc modulates aluminium-induced oxidative stress and cellular injury in rat brain. Metallomics 6 (10), 1941–1950. https://doi.org/10.1039/c4mt00097h.

Singla, N., Dhawan, D.K., 2017. Zinc Improves Cognitive and Neuronal Dysfunction During Aluminium-Induced Neurodegeneration. Mol Neurobiol.; 54(1):406-422. DOI: 10.1007/s12035-015-9653-9.

Thannickal, T.C., Lai, Y.Y., Siegel, J.M., 2007. Hypocretin (orexin) cell loss in Parkinson's disease. Brain.; 130(Pt 6):1586-95. DOI: 10.1093/brain/awm097.

Tierney, K.B., 2011. Behavioural assessments of neurotoxic effects and neurodegeneration in zebrafish. Biochim. Biophys. Acta; 1812(3):381-9. DOI: 10.1016/j.bbadis.2010.10.011.

Uversky, V.N., Li, J., Fink, A.L., 2001. Metal-triggered structural transformations, aggregation, and fibrillation of human alpha-synuclein. A possible molecular NK between Parkinson's disease and heavy metal exposure. J Biol Chem.; 276(47):44284-96. DOI: 10.1074/jbc.M105343200.

Way, S.W., Podojil, J.R., Clayton, B.L., Zaremba, A., Collins, T.L., Kunjamma, R.B., Robinson, A.P., Brugarolas, P., Miller, R.H., Miller, S.D., Popko, B., 2015. Pharmaceutical integrated stress response enhancement protects oligodendrocytes and provides a potential multiple sclerosis therapeutic. Nat Commun.; 6:6532. DOI: 10.1038/ncomms7532.

Westerfield, M., 2000. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), fourth ed. University of Oregon Press, Eugene.

Zatta, P., Lucchini, R., van Rensburg, S.J., Taylor, A., 2003. The role of metals in neurodegenerative processes: aluminum, manganese, and zinc. Brain Res. Bull.; 62(1):15-28. DOI: 10.1016/s0361-9230(03)00182-5.

Zhou, H., Chen, L., Gao, X., Luo, B., Chen, J., 2012. Moderate traumatic brain injury triggers rapid necrotic death of immature neurons in the hippocampus. J Neuropathol. Exp. Neurol.; 71(4):348-59. DOI: 10.1097/NEN.0b013e31824ea078.

Histological alterations and oxidative stress releveled in muscle of adult zebrafish after aluminium exposure

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Abstract

Oxidative stress reflects an imbalance between production and accumulation of oxygen reactive species (ROS) and the ability of antioxidant system to detoxify or repair the cellular damage induced. This process has been supposed as the mechanism of toxicity by various pollutants including aluminium. Aluminium is among the most abundant metals in nature, and its presence in the environment is further increasing by anthropogenic activities. In water bodies, the Al concentrations ranged from 0.001 until to 50 mg/L, raising concerns on health of aquatic organisms. For this reason, zebrafish was chosen as the model since is well suited for ecotoxicological studies and adult specimens were exposed to 11 mg/L of Al for 10, 15 and 20 days to assess both the morphology and the oxidative state of muscle tissue, considering the involvement of ROS, the activity of the main antioxidant enzymes, metallothioneins contents, but also oxidative damage and enzymes involved in energy consumption and in neuromuscular transmission. Collected data showed that after short-term exposures the treatment induced grave morphological alterations, activation of anaerobic metabolism and the increased activity of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and glutathione S-transferases. However, these effects stabilized with increasing exposure time. Moreover, only after 20 days of treatment did the oxidative damage to the proteins and the

activity of Acetylcholinesterase increase while the levels of metallothioneins were lower for all treated compared to control group. The overall information obtained gives a realistic snapshot of the potential pollution risk exerted by aluminium for the aquatic organisms and helps to provide new information of the mechanism of toxicity induced by this metal.

Keywords: Aerobic/anaerobic metabolism, Antioxidant enzymes, *Danio rerio,* Metallothioneins, Neuromuscular transmission, Oxidative biomarkers.

1. Introduction

Oxidative stress is defined as a perturbation of cell redox balance, that can be counteracted by the action of antioxidants (Pisoschi and Pop, 2015). The unbalance between the oxidant species and the antioxidant defense system may trigger several factors responsible for oxidative damage in the cell (Pizzino et al., 2017). The increase of oxidative stress is often associated to alteration of biological processes and to the onset of several pathologies (Poljsak et al., 2013). Moreover, the alteration of the oxidative state has been supposed as the mechanism of action by which various exogenous agents, like metals, pesticides and xenobiotics, induce toxicity (López et al., 2006; Rock and Patisaul, 2018). Aluminium (Al) is a metal highly widespread in the environment (Sharma et al., 2020), whose concentration, especially in the aquatic compartment, is further increased due to the phenomenon of acid rain, that, changing the acidity of the water, facilitate the solubilisation of the inorganic Al (Lawrence et al., 2007). This means that its concentration within the waters is highly variable from values of 0.001-0.05 mg/L when the pH is near-neutral values (WHO, 1997, 2013) up to 50 mg/L in polluted surface waters (ATSDR, 2008).

This metal can induce several toxic effects, depending on the concentration and the exposure time in both terrestrial and aquatic organisms (Alstad et al., 2005; Coleman et al., 2010). In particular, for aquatic organisms, it is known that Al can alter the survival and growth of several organisms both vertebrate and invertebrate (Cardwell et al., 2018; Gensemer et al., 2018; Monaco et al., 2017a). It can affect reproductive (Yokel, 2020) and swimming (Capriello et al., 2019, 2021a; Kumar and Gill, 2009) activity, but also induce histopathological alterations (Poléo et al., 2017). Given the evident danger of this metal for the health of aquatic species, the effect of Al toxicity induced in the muscle was investigated using *Danio rerio*, commonly called zebrafish, a known aquatic organism, very useful for screening of ecotoxicological effects (Favorito et al., 2011) induced by environmental pollutants (Fèlix et al., 2016; Santos et al., 2017) like metals (Monaco et al., 2016; 2017b), dyes (Capriello et al., 2021b; Motta et al., 2019) or herbicides (Lanzarin et al., 2019).

In particular, having evaluated in a previous study the induced alterations of Al exposure both on behavioural activity and on the oxidative state of the nervous system (Capriello et al., 2021a), this research aimed to investigate on the effects induced by this exposure on muscle tissue, in order to have a complete picture of the toxic action of Al, given that the musculature is a very important component to support the swimming performance. For this reason, the adult zebrafish organisms were exposed to the same concentration and with the same modalities as previously used (0.1 g/L of $AlCl_3 \cdot 6H_2O$ that corresponds to 11 mg/L of Al) and the effects on the muscles were evaluated on the same days, respectively after 10, 15 and 20 days (Capriello et al., 2021). As before, the concentration used is higher than that normally found in surface waters (WHO, 1997, 2021), to simulate what can happen in an aquatic environment polluted by an excessive presence of Al. However, even in this case, it was chosen a concentration that was sub-lethal for the organisms used, as demonstrated in a previously study (Monaco et al., 2017a).

In this study it was analysed the histology of the muscle of both red and white fibres and particular attention was paid to aspect related to oxidative state of this tissue. Specifically, it was assessed the involvement of reactive oxygen species (ROS) and different oxidative biomarkers: antioxidants

activity, enzymes involved in aerobic and anaerobic metabolism, oxidative damage induced to proteins and lipids but also levels of metallothioneins (MTs) and activity of acetylcholinesterase, a key functional protein of the neuromuscular junction.

Overall, this study gives a realistic snapshot of Al toxicity and provides new information on the mechanism of action of this metal, as widespread as dangerous.

2. Materials and methods

2.1 Zebrafish breeding

Adults zebrafish were maintained at the Department of Biology of the University of Naples "Federico II", under a natural photoperiod of 12:12 h light/dark, at temperature of 28.5 °C and pH of 7.5. Fish were fed twice a day with a commercial diet (Westerfield, 2000). Each organism, before being sacrificed, was euthanized with an overdose of ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma Aldrich, Germany) to minimize suffering. All used protocols were in accordance with the local regulation and international guidelines (Directive 2010/63/EU) and approved by Italian Ministry of Health (Permit Number: 147/2019-PR).

2.2 Chemicals and treatment

The treatment solution of 400 μM (0.1 g/L) of $AlCl_3 \cdot 6H_2O$, was prepared dissolving 2.5 g of $AlCl_3 \cdot 6H_2O$ (Carlo Erba, Italy; CAS no. 7784-13-6), in 25 L fish tank filled with breeding solution (1/3 bidistilled water, 2/3 tap water).

Three groups with each 10 healthy zebrafish of about 6 months were placed in a 25 L tanks and exposed respectively for 10, 15 and 20 days to Al solution (11 mg/L of Al). Another group of 10 fish, exposed only to breeding solution, was used as a control group. Each treatment was replayed for three times.

Every day, the total treatment solution was changed, the food debris was removed and the parameters of temperature and pH were checked and maintained constant (28.5 °C, pH 7.5).

At the end of each experimental period, all fish were sacrificed and divided for the several analyses. For histological analysis, three muscle tissue samples were taken both from each treatment group and from control group. From the same groups, four muscle tissue samples were also collected for can carry out biochemical biomarkers' analysis; and other three muscle tissue samples for metallothioneins assay. This selection was made for each independent replicate used.

2.3 Histological analysis

Standard histological protocols for optical microscopy were carried out for all samples. The samples were fixed in Bouin solution for 48 h to preserve the protoplasmic structure, dehydrated in graded series of ethanol and included in paraffin. Subsequently, sections of 5 μm cut with the rotary microtome, were deparaffinized, hydrated, and stained by trichrome of Mallory (Capriello et al., 2021b; Carotenuto et al., 2020). At the end of staining, the sections were clarified in xylene and mounted in resin for microscopic examination and the images were acquired by the light microscope (Zeiss, Germany).

2.4 Protein determination and sample collection

At the end of the experimental period, samples were collected separately, homogenized and processed as described in our previous study (Capriello et al., 2021a; Félix et al., 2018). Protein determination of all samples, was performed using the BioTek's Take3™ Micro-Volume Plate by optical absorbance at 280 nm using bovine serum albumin (BSA) as standard. All aliquots were stored at -20°C until analysis.

2.5 Determination of ROS accumulation and enzyme activities

ROS accumulation and the activity of all enzymes were performed in duplicate and measured against a reagent blank. All analyses were carried out by known experimental procedures, described in detail in our previous works (Capriello et al., 2021a; Félix et al., 2018) and briefly reported here. ROS accumulation was estimated at 485 nm and 530 nm excitation and emission wavelengths, respectively, by using a fluorescent probe (dichlorofluorescein diacetate, DCFH-DA) in a Varian

Cary Eclipse spectrofluorometer (Varian, USA) equipped with microplate reader. The reduced (GSH) and oxidized (GSSG) glutathione levels were evaluated fluorometrically, at 320 nm and 420 nm, respectively excitation and emission wavelengths with the fluorochrome ortho-phthalaldehyde (OPT) in the same spectrofluorometer. The oxidative-stress index (OSI) was calculated according to the quotient between GSH and GSSG. All subsequent enzymatic assays were carried out with PowerWave XS2 microplate scanning spectrophotometer (Bio-Tek Instruments, USA) at 30° C unless otherwise specified. The superoxide dismutase (SOD) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT) at 560 nm. Catalase (CAT) activity was determined at 240 nm following the decrease in absorbance of H_2O_2. Glutathione peroxidase (GPx) activity was determined by decrease in absorbance of NADPH at 340 nm. To evaluate the activity of glutathione S-transferases (GST), CDNB (1-Chloro-2,4-nitrobenzene) complexation was measured at 340 nm. To determine the total ATPase activity, the inorganic phosphate present in the reaction media was measured at a wavelength of 820 nm. For the lactate dehydrogenase (LDH) activity, the decrease in the absorbance due to the oxidation of NADH was monitored at 340 nm. The activity of acetylcholinesterase (AChE) enzyme, one of the key functional proteins of the neuromuscular junction, was analysed by following the increase in the absorbance due to the conversion of acetylthiocholine to thiocholine by DTNB at 405 nm.

2.6 Metallothioneins assay and oxidative damage biomarkers

To metallothioneins assay, a solution (1:13 absolute ethanol and chloroform) was added to supernant of samples' homogenates and centrifuged (4 °C) at 6000 xg for 10 min. After some wash and centrifugation necessary to metallothionein purification, the dried pellet was resuspended in Ellman's solution ((NaCl 2 M; 5,5'-d ithiobis (nitrobenzoicacid) 43 mM in phosphate buffer 0.2 M, pH 8)), adding EDTA-HCl and NaCl. The concentration of metallothioneins was evaluated by reading the absorbance at 412 nm. The lipid peroxidation (TBARS) was determined by a thiobarbituric (TBA) acid-based at 530 nm. The oxidative damage to proteins (carbonyls, CO) was evaluated by the DNPH

(2,4-dinitrophenylhydrazine) method at 450 nm. All analyses were carried out by known experimental procedures, described in detail in our previous works (Capriello et al., 2021a; Félix et al., 2018) and briefly reported here.

2.7 Statistical analysis

A sample size calculation was performed with the G*Power 3 (University of Düsseldorf, Germany) based on standard deviations (Oliveira et al., 2013). All data were checked for normality and homogeneity by Shapiro-Wilk test and Brown-Forsythe test, respectively before evaluating the statistical significance. One-way analysis of variance ANOVA followed by Tukey's pairwise comparison tests was used to evaluate the differences among treatments. Graphs and statistical analysis were processed using GraphPad-Prism 7 (GraphPad Software, La Jolla California USA, www.graphpad.com). The data are expressed as mean±SD and were considered to be statistically significant at $p<0.05$. Different lowercase letters used indicate significant differences between groups.

3. Results

3.1 Muscle morphology

Skeletal muscle in the zebrafish, like in other fish (Avallone et al.,2015; Carani et al., 2013) are organized in compartments: the red layer, just under the skin, composed of slow-twitch fibres, and the deep white layer, formed by fast-twitch glycolytic fibres. Morphological analysis of musculature of the control organisms showed highly compact and well-organized tissue. Both the red (Fig. 1A) and white fibres (Fig. 1E) showed a regular and compact arrangement of myofilaments with peripheral nuclei and a thin layer of connective tissue, usually called endomysium, to separate the bundles of fibres (myotomes). In the treated organisms there was not only an alteration of the staining affinity, especially in the red fibres, but also an increase in the thickness of the endomysium. In particular, the red fibres of the organisms exposed for 10 days were highly disorganized with resorbed myofibrils (Fig.1B) than those of the controls. Also, the white fibres were altered and showed an

increase of connective tissue, that made the reticular organization of connective fibres separating muscular masses more visible (Fig. 1F). Organisms exposed to 15 days of treatment had red fibres more similar to those of the controls, less spaced although, in some point multinucleated (Fig. 1C). Instead, the white fibres appeared enclosed in larger and more compact myotomes, with a decrease in the thickness of the endomysium (Fig. 1G) compared to the other Al-treated. Also, the red fibres of the organisms exposed to Al for 20 days showed a fairly compact structure although in some points they were multinucleated and spaced apart (Fig. 1D). Instead their white fibres while presenting an endomysium still abundant, appeared smaller than those of organisms exposed for 15 days, and more similar to those found in control tissue (Fig. 1H).

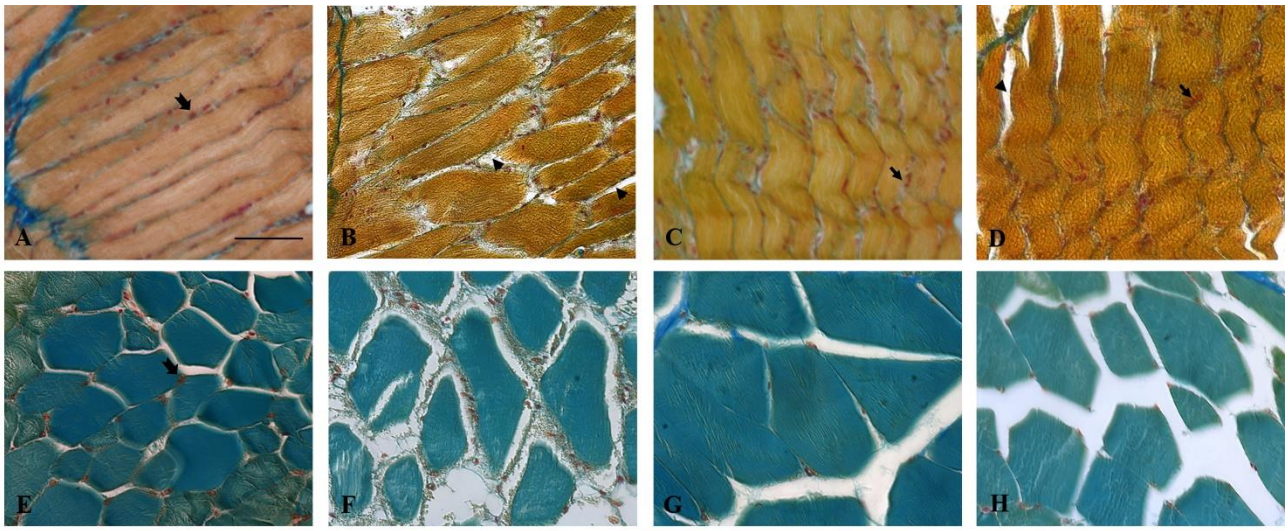

Fig. 1 Muscle tissue of zebrafish, sections stained by Mallory trichrome staining. (A-D): red muscle; (E-F): white muscle. (A, E) Control group with a tissue well conserved, ordered fibres with peripheral nuclei (arrow➡). (B, F) Fibres of organisms exposed to Al for 10 days, with increase in the thickness of the endomysium and resorbed myofibrils (arrow ▶). (C, G) Organisms exposed to Al for 15 days with red fibres less spaced, although multinucleated (arrow ↘) and the white fibres enclosed in larger and more compact myotomes. (D, H) Organisms exposed to Al for 20 days, with red muscle more compact, with few fibres multinucleated and separated by spaces. The white fibres while presenting an endomysium still abundant, showed smaller myotomes, similar to control ones. Scale bar: 50 µm**.**

3.2 *ROS accumulation and enzyme activities*

ROS accumulation showed no significant differences between treatments (Fig. 2A). The CAT activity, presented no significant changes between the different treatment groups (Fig. 2B), while other enzyme activities changed due to exposed to Al with a variable trend, depending on the

considered enzyme. SOD activity increased significantly in the muscle tissue of organisms exposed to Al for 10 days ($p<0.01$) compared to control group but drastically decreased for subsequent exposure times after 15 days of exposure ($p<0.01$) and after 20 days of exposure, even reaching lower levels than those of controls (Fig. 2C). Instead, the values of GPx activity (Fig. 2D) registered for the organisms exposed to AlC_3 for 10,15, and 20 days increased significantly ($p<0.05$) relative to the control group. Similarly, for GST activity, the control values were significantly lower than the values registered in the Al-treated at increasing times ($p<0.05$; $p<0.05$; and $p<0.01$, respectively). The reduced glutathione levels (GSH) (Fig. 3A), was approximately 3-fold lower in the muscular tissue of organisms exposed to Al for 10 days ($p<0.05$) compared to the control group and tended to return to values similar to those of control group, for longer exposure times. The oxidized glutathione levels (GSSG) (Fig. 3B), followed a similar trend to GSH activity, decreased for the specimens exposed to Al for 10 days ($p<0.05$) compared to the control group and tended to increase at prolonged exposure times with values similar to those of the control group. The oxidative stress index (OSI), showed no significant differences between control and treated organisms (Fig. 3C). The Pi production by ATPases significantly increased in muscle tissue of fish exposed to treatment for 10 days ($p<0.05$) compared to the control group. Fish exposed for 15 days showed levels of Pi similar to the control group, while after 20 days of exposure the organisms showed levels significantly different from those of the control group ($p <0.05$) and more similar to those recorded in the organisms exposed to Al for 10 days (Fig. 3D).

The LDH activity was significantly increased to all treated groups ($p<0.01$; $p<0.001$ and $p<0.001$ for 10, 15 and 20 days of exposure, respectively) (Fig. 3E) compared to values found in the control organisms. Instead, acetylcholinesterase (AChE) showed a significantly increased only in organisms exposed to the maximum exposure time (20 days, $p<0.001$) compared to the control group and other Al-treated for less time (Fig. 3F).

3.3 Metallothioneins assay and biomarkers of oxidative damage

The quantity of metallothioneins (MTs) (Fig. 3G), significantly decreased in muscular tissue of all organisms exposed to Al ($p<0.001$) for all the organisms treated in comparison to the control group). The lipid peroxidation (TBARS) levels (Fig. 3H), were lower in the organisms exposed for 10 days ($p<0.01$) than those recorded in the control group. This difference remained significant for organisms exposed to 15 days of treatment ($p<0.05$), while organisms exposed to 20 days showed levels more similar to physiological ones. Conversely, the oxidative damage of proteins (carbonyls, CO) (Fig. 3I), showed a significant increase only after 20 days of exposure when compared with the values of the control group ($p<0.05$).

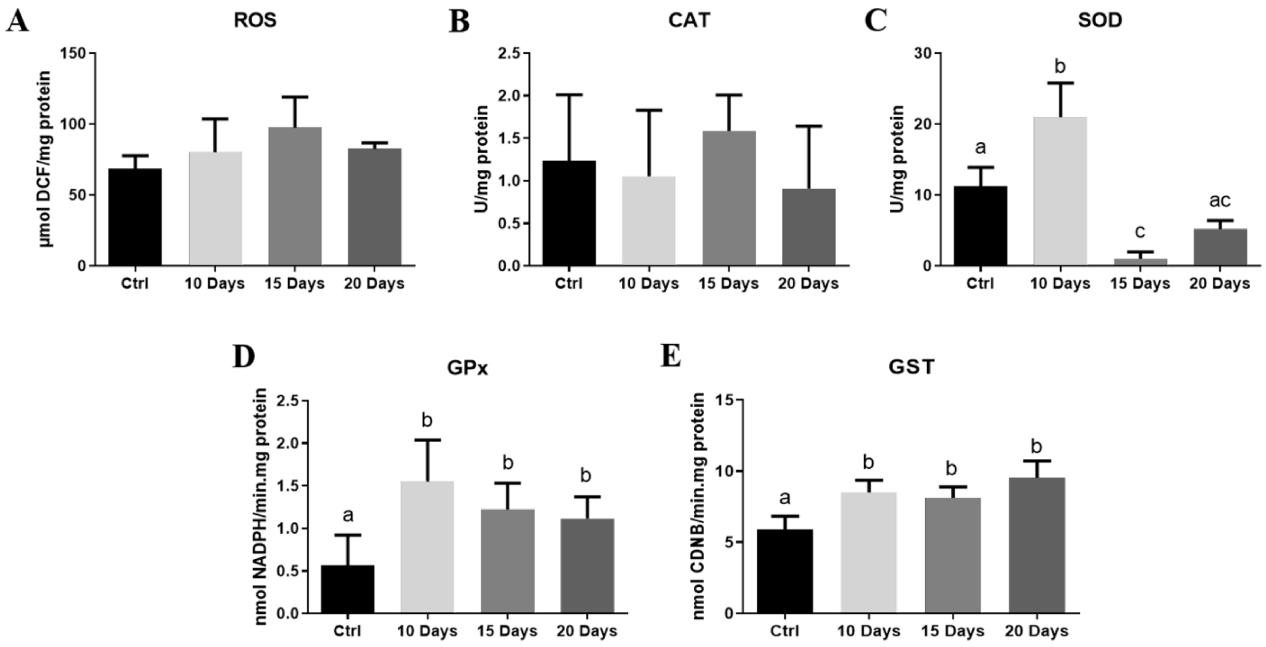

Fig. 2 (A) Reactive oxygen species accumulation (ROS), (B) catalase (CAT) activity, (C) superoxide dismutase (SOD) activity, (D) glutathione peroxidase (GPx) activity, glutathione S-transferases (GST) activity, in the muscular tissue of fish exposed to aluminium (11 mg/L Al) during 10, 15 and 20 days and compared to control (Ctrl). Data are expressed as mean±SD. Different lowercase letters indicate significant differences between groups.

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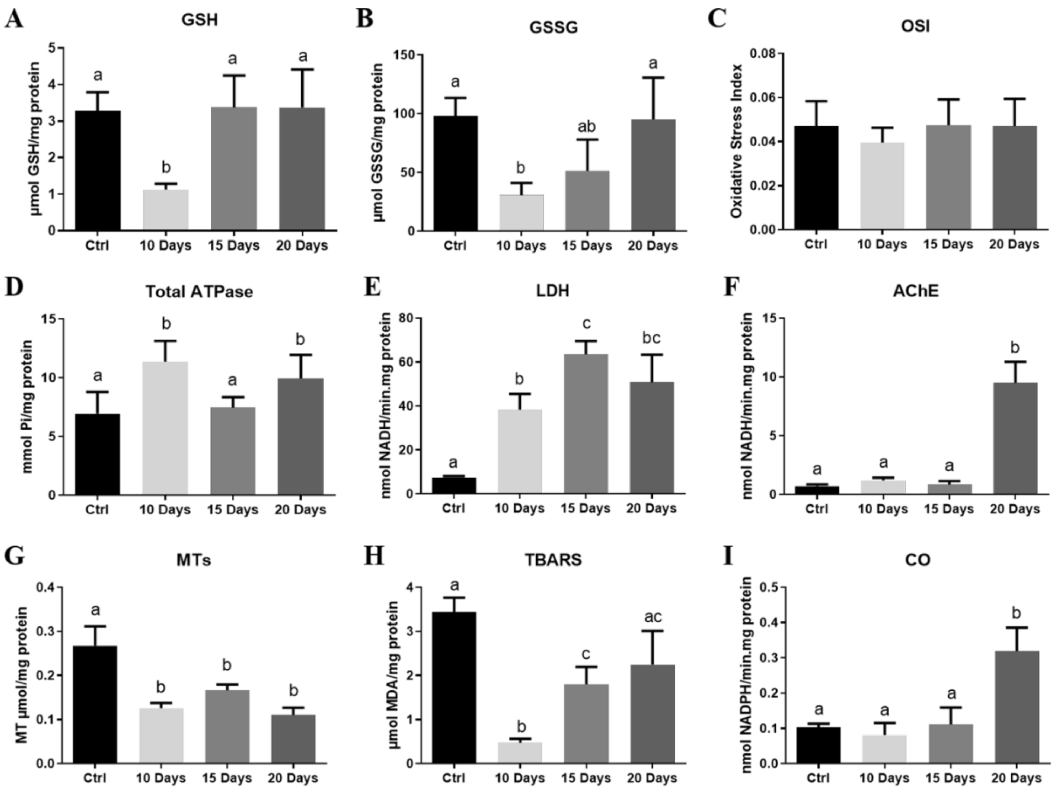

Fig. 3 (A) Levels of reduced glutathione (GSH) and (B) oxidized glutathione (GSSG), (C) oxidative stress index (OSI), (D) total ATPase activity, (E) lactate dehydrogenase (LDH) activity, (F) acetylcholinesterase (AChE) activity, (G) metallothioneins (MTs) levels, (H) lipid peroxidation (TBARS) and (I) oxidative damage to proteins (carbonyls, CO) in the muscular tissue of fish exposed to aluminium (11 mg/L Al) during 10, 15 and 20 days and compared to control (Ctrl). Data are expressed as mean±SD. Different lowercase letters indicate significant differences between groups.

4. Discussion

The aim of this work was to evaluate the effects of adult zebrafish exposed to 11mg/L Al for 10, 15 and 20 days on muscle tissue, as a continuation of a previous study in which the toxic effect of this compound on neurobehavioral activity and on the oxidative state of the brain was demonstrated (Capriello et al., 2021a). In particular, with regard to swimming activity and behavioural response, there was a deterioration of the parameters measured in organisms exposed to Al for a short time and a stabilization/improvement of these parameters after a longer period of exposure (20 days). This

stabilization was sometimes reflected in the trend of the parameters used to evaluate the oxidative state of the brain. In light of this, it therefore seemed necessary to analyse the effects induced by Al on muscle tissue, given its important involvement in swimming behaviour.

Based on the histological analysis data, a high alteration of muscle tissue is evident in organisms exposed for less time to treatment, while an improvement in morphological conditions was present in organisms exposed for longer times. Morphological alterations of muscle tissue are a common symptom of suffering, found in zebrafish after exposure to various metals like cadmium or silver (Avallone et a., 2015; Sayed et al., 2020). In particular, the organisms exposed to Al for 10 days showed resorption of myofibrils. This phenomenon has already been evidenced in the zebrafish musculature after exposure to cadmium (Avallone et al., 2015). Probably, as assumed for the latter case, it is possible to hypothesize the involvement of Al in the activation of enzymes involved in myofibril degradation such as caspase-3 or calpains (Smuder et al., 2010). Another explanation could be the interaction of Al with the physiological process of fibril replacement since the resorption of fibre was evident in outermost part of the fibre where turnover usually occurs (Neti el al., 2009). After 15 days of treatment, the white musculature appeared formed by myotomes larger than those found for other treatments or in controls. It is known that white muscle contracts faster and more efficiently than red muscle (Wu et al., 2018), so the change that occurred in Al-treated for 15 days supports the previously recorded motility data (Capriello et al., 2021a). Probably an increase in white fibres was needed to support the increased swimming activity of these organisms. After 20 days, on the other hand, the morphology of both the white and the red muscles, while still presenting small alterations, approached the physiological one. This improvement, also found in the musculature after high exposure to cadmium (Avallone et al., 2015), could be linked to the process of muscle regeneration after a highly conserved insult in zebrafish (Montandon et al., 2021).

Oxidative stress is a known important factor in the pathogenesis of many abnormalities and tissue alterations (Pisoschi and Pop, 2015). In this case, the amount of ROS did not vary significantly between Al-treated organisms and those controls, probably due to the activation of the antioxidant

system, designed to protect the tissue from oxidative insults (Pisoschi and Pop, 2015). Indeed, the antioxidant enzyme SOD, was already significantly higher in the muscle tissue of the organisms exposed to treatment for 10 days, because it is the first enzyme to respond as antioxidant defense to neutralize the amount ROS (Yang et al., 2014). Instead, for the longer exposure times its decrease was evident, probably due to the intervention of other enzymes and more efficient antioxidant systems such as GPx and GST, whose activity is high and constant for all treatment periods compared to that recorded in the control. In particular, GST is an important detoxicant enzyme within phase II xenobiotic metabolism, which may play an important role in Al detoxification, since it catalyses the synthetic conjugation reactions of electrophilic compounds as Al^{3+} to GSH (Cappello et al., 2016).

In light of this, the levels of GSH and its oxidized form (GSSG) were also altered by Al exposure. In particular, both showed a similar trend with a sharp decrease in their levels after 10 days of treatment, and a recovery for longer exposure times (15 and 20 days), when they showed physiological enzymatic levels, not different from those of the controls. This reduction is in agreement with the data reported in zebrafish after exposure to various toxic agents such as arsenic (Adeyemi et al., 2015), cadmium or chromium (Jin et al., 2015), but also in rat after exposure to Al (Singla and Dhawan, 2014). Probably, this depleted is due to GSH that rapidly binds to the produced reactive oxygen species, thus detoxifying them (Massarsky et al., 2017). Also, the increase in GSH levels, for later times, with a U-shape response, is reflected in another study conducted on zebrafish, treated with antidepressant amitriptyline (Yang et al., 2014). Despite the changes observed, the OSI was not affected reflecting a “normal” oxidative environment. This phenomenon is probably due to the activation of the antioxidant mechanism efficiency.

Lastly, also levels of MTs, specialized scavengers for the removal of toxic metal ions (Chen et al., 2014), were lower for all treated than for control group; probably because, as occurred in other studies (Barka et al., 2001; De Smet et al., 2001), metal accumulation might have compromised the MT biosynthesis.

Instead, analysing the activity of the enzymes involved in energy consumption, the ATPase, that provide the primary energy source for the majority of cell functions (Félix et al., 2016), increased in the muscle tissue of organisms exposed to Al for 10 days, decreased for those exposed to 15, returning to levels similar to those of the control group, and increased again for organisms exposed to 20 days of treatment. LDH showed an almost opposite trend, while remaining higher in all treated than in the control, showed a significantly higher peak than the other treatment periods after 15 days. It must be said that the LDH is an important factor in muscular physiology (Silva Santos et al., 2018) in the ability of some fish to produce bursts of swimming (Osman et al., 2010). Moreover, in case of oxidative stress, a common response is metabolic hypoxia, which can increase the anaerobic pathway of energy production and consequently the LDH activity (Oliveira et al., 2016). This suggests, in conjunction with the increase in locomotor activity previously assessed (Capriello et al., 2021a), the pyruvate, the final product of glycolysis, was preferentially used to produce lactate to cope with the high demand of energy under toxic stress (Frasco and Guilhermino, 2002). Although it is less efficient, anaerobic glycolysis is 100 times faster than oxidative phosphorylation and enable to fulfil energy requirements in short-term (Valvona et al., 2016). Furthermore, elevation of LDH in Al-treated for 15 days with the consequent bias towards the anaerobic glycolytic pathway is in agreement with the increase in white fibres recorded at these times, which primarily use anaerobic glycolysis as their ATP source (Ceaser and Hunter, 2015).

Our experiments showed that Al was able to increase AChE activity only after the maximum exposure time. AChE has the task of removing acetylcholine (ACh), a fundamental neurotransmitter for neuromuscular activity, once released from the presynaptic nerve terminals (Senger et al., 2011). This increase is in accord with other studies conducted to evaluate the toxicity of Al (Kaizer et al. 2005; Senger et al., 2011), which also report an increase in AChE activity extending after long-term exposure to Al. Probably, as they suggest, Al could induce changes in the conformational state of the AChE molecule, responsible for the induction of AChE activity observed.

Oxidative stress can determine structure modifications and function modulation in lipids and proteins (Pisoschi and Pop, 2015). In this case, the levels of TBARS, manifestation of lipid oxidative damage (Souza et al., 2019), was lower in the treated than in the control group but tended to normalize with increasing exposure period. This phenomenon, albeit in contrast with the usual toxicity-response trend, has already been seen in zebrafish and in other fish after exposure to different pollutants (Cappello et al., 2016; Praskova et al., 2014) and could be due to the evident activation of the antioxidant defenses. Indeed, these defenses respond to conditions of increased oxidative stress with a compensatory mechanism for the overproduction of free radicals (Souza et al., 2019). This may explain the decrease in lipid peroxidation level. On the other hand, CO levels increased only after 20 days of treatment, probably because they are more stable compared to lipid peroxidation. In fact, often they are detected only in conditions of considerable oxidative stress (Levine, 2002; Mezzomo et al., 2019; Shi et al., 2005). Overall, taken together the results show that the prolonged effect of Al induced an improvement/stabilization of the conditions of the muscle tissue, both from the point of view of the oxidative state and of the tissue morphology. This phenomenon supports the data previously detected in the brain and is in agreement with the data relating to behaviour and swimming capacity (Capriello et al., 2021a). Furthermore, it must be remembered that this phenomenon is not uncommon in zebrafish when such specimens are exposed to pollutants for prolonged periods or high concentrations, probably because such exposures stimulate detoxification or adaptation more efficient (Acosta et al., 2016; Avallone et al., 2015; Pilehvar et al., 2020).

5. Conclusion

All the information presented in this study helped to elucidate the possible mechanism of Al-induced toxicity in fish muscle. Specifically, from our observations, it can be concluded that short periods induce alteration of the tissue morphology of both red and white fibres, increase anaerobic metabolism and activation of the antioxidant system, necessary to reduce the effects of ROS.

However, long exposures induce improvement/stabilization of the conditions probably due to the activation of an adaptive response that allows zebrafish to survive in less favourable conditions. Overall, the biochemical and histological changes in the muscular tissue induced by Al exposure might represent a relevant contribution to understanding the effects of the increasing diffusion of this metal within the aquatic compartment.

Conflict of interest

The authors declare that they have no financial interests or personal relationships that could influence the content of the paper.

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References

Acosta, D.D.S., Danielle, N.M., Altenhofen, S., Luzardo, M.D., Costa, P.G., Bianchini, A., Bonan, C.D., da Silva, R.S., Dafre, A.L., 2016. Copper at low levels impairs memory of adult zebrafish (Danio rerio) and affects swimming performance of larvae. Comp. Biochem. Physiol. C Toxicol. Pharmacol.; 185-186:122-130. DOI: 10.1016/j.cbpc.2016.03.008.

Adeyemi, J.A., da Cunha Martins-Junior, A., Barbosa, F. Jr., 2015. Teratogenicity, genotoxicity and oxidative stress in zebrafish embryos (Danio rerio) co-exposed to arsenic and atrazine. Comp. Biochem. Physiol. C Toxicol.; 172-173,7–12. DOI:10.1016/j.cbpc.2015.04.001.

Agency for Toxic Substances and Disease (ATSDR), 2008. Toxicological Profile for Aluminum. CAS, 7429-90-95.

Alstad, N.E., Kjelsberg, B.M., Vøllestad, L.A., Lydersen, E., Pol´eo, A.B., 2005. The significance of water ionic strength on aluminium toxicity in brown trout (Salmo trutta L.). Environ. Pollut. 133 (2), 333–342. https://doi.org/10.1016/j.envpol.2004.05.030.

Avallone, B., Agnisola, C., Cerciello, R., Panzuto, R., Simoniello, P., Cretì, P., Motta, C.M., 2015. Structural and functional changes in the zebrafish (Danio rerio) skeletal muscle after cadmium exposure. Cell Biol. Toxicol.; 31(6):273-83. DOI: 10.1007/s10565-015-9310-0.

Barka, S., Pavillon, J., Amiard, J., 2001. Influence of different essential and non-essential metals on MTLP levels in the Copepod Tigriopus brevicornis. Comp. Biochem. Physiol. C Toxicol. Pharmacol.; 128(4):479-93. DOI: 10.1016/s1532-0456(00)00198-8.

Cappello, T., Brandão, F., Guilherme, S., Santos, M.A., Maisano, M., Mauceri, A., Canário, J., Pacheco, M., Pereira, P., 2016. Insights into the mechanisms underlying mercury-induced oxidative stress in gills of wild

fish (Liza aurata) combining (1)H NMR metabolomics and conventional biochemical assays. STOTEN, 548-549,13–24. DOI:10.1016/j.scitotenv.2016.01.008.

Capriello, T., Félix, L.M., Monteiro, S.M., Santos, D., Cofone, R., Ferrandino, I., 2021a. Exposure to aluminium causes behavioural alterations and oxidative stress in the brain of adult zebrafish. Environ. Toxicol. Pharmacol.; 85:103636. DOI: 10.1016/j.etap.2021.103636.

Capriello, T., Grimaldi, M.C., Cofone, R., D'Aniello, S., Ferrandino, I., 2019. Effects of aluminium and cadmium on hatching and swimming ability in developing zebrafish. Chemosphere 222, 243–249. https://doi.org/10.1016/j.chemosphere.2019.01.140

Capriello, T., Visone, I.M., Motta, C.M., Ferrandino, I., 2021b. Adverse effects of E150d on zebrafish development. Food Chem. Toxicol; 147:111877. DOI: 10.1016/j.fct.2020.111877.

Carani, F.R., Duran, B.O.D.S., Paula, T.G.D., Piedade, W.P. and Dal-Pai-Silva, M., 2013. Morphology and expression of genes related to skeletal muscle growth in juveniles of pirarucu (Arapaima gigas, Arapaimatidae, Teleostei). Acta Scientiarum. Animal Sciences; 35(3): 219-226. https://doi.org/10.4025/actascianimsci.v35i3.18219.

Cardwell, A.S., Adams, W.J., Gensemer, R.W., Nordheim, E., Santore, R.C., Ryan, A.C., Stubblefield, W.A., 2018. Chronic toxicity of aluminum, at a pH of 6, tofreshwater organisms: Empirical data for the development of interna-tional regulatory standards/criteria. Environ. Toxicol. Chem.; 37:36–48. DOI:10.1002/etc.3901.

Carotenuto, R., Capriello, T., Cofone, R., Galdiero, G., Fogliano, C., Ferrandino, I., 2020. Impact of copper in Xenopus laevis liver: histological damages and atp7b downregulation. Ecotoxicol. Environ. Saf. 30, 188–109940. https://doi.org/10.1016/j.ecoenv.2019.109940.

Ceaser, T., Hunter, G., 2015. Black and White race differences in aerobic capacity, muscle fiber type, and their influence on metabolic processes. Sports Med.; 45(5):615-23. DOI: 10.1007/s40279-015-0318-7.

Chen, L., Ma, L., Bai, Q., , Zhu, X., Zhang, J., Wei , Q., Li, D., Gao, C., Li, J., Zhang, Z., Liu, C., He, Z., Zeng, X., Zhang, A., Qu, W., Zhuang, Z., Chen, W., Xiao, Y., 2014. Heavy metal-induced metallothionein expression is regulated by specific protein phosphatase 2A complexes. J. Biol. Chem.; 289(32), 22413–22426. DOI:10.1074/jbc.M114.548677.

Coleman, J.G., Johnson, D.R., Stanley, J.K., Bednar, A.J., Weiss Jr., C.A., Boyd, R.E., Steevens, J.A., 2010. Assessing the fate and effects of nano aluminum oxide in the terrestrial earthworm, Eisenia fetida. Environ. Toxicol. Chem. 29 (7), 1575–1580. https://doi.org/10.1002/etc.196, 2010.

De Smet, H., De Wachter, B., Lobinski, R., Blust, R., 2001. Dynamics of (Cd,Zn)-metallothioneins in gills, liver and kidney of common carp Cyprinus carpio during cadmium exposure. Aquat. Toxicol.; 52, 269–281.DOI: 10.1016/S0166-445X(00)00136-3.

Favorito, R., Chiarelli, G., Grimaldi, M.C., De Bonis, S., Lancieri, M., Ferrandino, I., 2011. Bioaccumulation of cadmium and its cytotoxic effect on zebrafish brain. Chem. Ecol.;, 27(2), 39-46. https://doi.org/10.1080/02757540.2011.625937.

Félix, L.M., Vidal, A.M., Serafim, C., Valentim, A.M., Antunes, L.M., Campos, S., Matos, M., Monteiro, S.M., Coimbra, A.M., 2016. Ketamine-induced oxidative stress at different developmental stages of zebrafish (Danio rerio) Embryos. R. Soc. Chem.; 6(66),61254-61266. DOI: 10.1039/c6ra08298j.

Félix, L.M., Vidal, A.M., Serafim, C., Valentim, A.M., Antunes, L.M., Monteiro, S.M., Matos, M., Ana, M., Coimbra, A.M., 2018. Ketamine induction of p53-dependent apoptosis and oxidative stress in zebrafish (Danio rerio) embryos. Chemosphere 201, 730–739. https://doi.org/10.1016/j.chemosphere.2018.03.049.

Frasco, M.F., Guilhermino, L., 2002. Effects of dimethoate and beta-naphthoflavone on selected biomarkers of Poecilia reticulata. Fish Physiol. Biochem.; 26, 149–156. https://doi.org/10.1023/A:1025457831923.

Gensemer, R.W., Gondek, J.C., Rodriquez, P.H., Arbildua, J.J., Stubblefield, W.A., Cardwell, A.S., Santore, R.C., Ryan, A.C., Adams, W.J., Nordheim, E., 2018. Evaluating the effects of pH, hardness, and dissolved organic carbon on the toxicity of aluminum to freshwater aquatic organisms under circumneutral conditions. Environ. Toxicol. Chem.; 37(1):49-60. DOI: 10.1002/etc.3920.

Jin, Y., Liu, Z., Liu, F., Ye, Y., Peng, T., Fu, Z., 2015. Embryonic exposure to cadmium (II) and chromium (VI) induce behavioral alterations, oxidative stress and immunotoxicity in zebrafish (Danio rerio). Neurotoxicol. Teratol.; 48, 9–17. DOI:10.1016/j.ntt.2015.01.002.

Kaizer, R.R., Corrêa, M.C., Spanevello, R.M., Morsch, V.M., Mazzanti, C.M., Gonçalves, J.F, Schetinger, M.R., 2005. Acetylcholinesterase activation and enhanced lipid peroxidation after long-term exposure to low levels of aluminum on different mouse brain regions. J. Inorg. Biochem.; 99(9):1865-70. DOI: 10.1016/j.jinorgbio.2005.06.015.

Kumar, V., Gill, K.D., 2009. Aluminium neurotoxicity: neurobehavioural and oxidative aspects. Arch. Toxicol.; 83: 965–978. https://doi.org/10.1007/s00204-009-0455-6.

Lanzarin, G.A.B., Félix, L.M., Santos, D., Venâncio, C.A.S., Monteiro, S.M., 2019. Dose-dependent effects of a glyphosate commercial formulation - Roundup® UltraMax - on the early zebrafish embryogenesis. Chemosphere, 223,514–522. DOI:10.1016/j.chemosphere.2019.02.071.

Lawrence, G.B., Sutherland, J.W., Boylen, C.W., Nierzwicki-Bauer, S.W., Momen, B., Baldigo, B.P., Simonin, H.A., 2007. Acid rain effects on aluminum mobilization clarified by inclusion of strong organic acids. Environ. Sci. Technol.; 41 (1), 93–98. https://doi.org/10.1021/es061437v.

Levine, R.L., 2002. Carbonyl modified proteins in cellular regulation, aging, and disease. Free Radic. Biol. Med.; 32(9):790-6. DOI: 10.1016/s0891-5849(02)00765-7.

López, E., Arce, C., Oset-Gasque, M.J., Cañadas, S., González, M.P., 2006. Cadmium induces reactive oxygen species generation and lipid peroxidation in cortical neurons in culture. Free Radic. Biol. Med.; 40(6):940-51. DOI: 10.1016/j.freeradbiomed.2005.10.062.

Massarsky, A., Kozal, J.S., Di Giulio, R.T., 2017.Glutathione and zebrafish: Old assays to address a current issue. Chemosphere, 168, 707-15. DOI: 10.1016/j.chemosphere.2016.11.004.

Mezzomo, N.J., Fontana, B.D., Müller, T.E., Duarte, T., Quadros, V.A., Canzian, J., Pompermaier, A., Soares, S.M., Koakoski, G., Loro, V.L., Rosemberg, D.B., Barcellos, L.J.G., 2019. Taurine modulates the stress response in zebrafish. Horm. Behav.; 109:44-52. DOI: 10.1016/j.yhbeh.2019.02.006.

Monaco, A., Capriello, T., Grimaldi, M.C., Schiano, V., Ferrandino I., 2017b. Neurodegeneration in zebrafish embryos and adults after cadmium exposure. Eur. J. Histochem.; 61(4), 19PMC5656802. DOI: 10.4081/ejh.2017.2833.

Monaco, A., Grimaldi, M.C., Ferrandino, I., 2016. Neuroglial alterations in the zebrafish brain exposed to cadmium chloride. J. Appl. Toxicol.; 36(12),1629-38. DOI: 10.1002/jat.3328.

Monaco, A., Grimaldi, M.C., Ferrandino, I., 2017a. Aluminium chloride-induced toxicity in zebrafish larvae. J. Fish Dis. 40 (5), 629–635. https://doi.org/10.1111/jfd.12544.

Montandon, M., Currie, P.D., Ruparelia, A.A., 2021. Examining Muscle Regeneration in Zebrafish Models of Muscle Disease. J. Vis. Exp.; (167). DOI: 10.3791/62071.

Motta, C.M., Simoniello, P., Arena, C., Capriello, T., Panzuto, R., Vitale, E., Agnisola, C., Tizzano, M., Avallone, B., Ferrandino, I., 2019. Effects of four food dyes on development of three model species, Cucumis sativus, Artemia salina and Danio rerio: assessment of potential risk for the environment. Environ. Pollut.; 253,1126-1135. https://doi.org/10.1016/j.envpol.2019.06.018.

Neti, G., Novak, S.M., Thompson, V.F., Goll, D.E., 2009. Properties of easily releasable myofilaments: are they the first step in myofibrillar protein turnover? Am. J. Physiol. Cell Physiol. 2; 296(6):C1383-90. DOI: 10.1152/ajpcell.00022.2009.

Oliveira, M., Teixeira, J.C., Vasconcelos-Nobrega, C., Félix, L.M., Sardao, V.A., Colaco, A.A., Oliveira, P.A., Peixoto, F.P., 2013. Mitochondrial and liver oxidative stress alterations induced by N-butyl-N-(4-hydroxybutyl)nitrosamine: relevance for hepatotoxicity. J. Appl. Toxicol. 33, 434–443. https://doi.org/10.1002/jat.1763.

Oliveira, R., Grisolia, C.K., Monteiro, M.S., Soares, A.M., Domingues, I., 2016. Multilevel assessment of ivermectin effects using different zebrafish life stages. Comp. Biochem. Physiol. C Toxicol. Pharmacol.; 187,50–61. DOI:10.1016/j.cbpc.2016.04.004.

Osman, A., Reheem, A., Baset, M.A. El, AbuelFadl, K., Rab, A., 2010. Enzymatic and histopathologic biomarkers as indicators of aquatic pollution in fishes. Nat. Sci. 2,1302–1311. DOI: 10.4236/ns.2010.211158.

Pilehvar, A., Town, R.M., Blust, R., 2020. The effect of copper on behaviour, memory, and associative learning ability of zebrafish (Danio rerio). Ecotoxicol. Environ. Saf.; 188-109900. DOI: 10.1016/j.ecoenv.2019.109900.

Pisoschi, A.M., Pop, A., 2015. The role of antioxidants in the chemistry of oxidative stress: A review. Eur J Med Chem.; 97:55-74. DOI: 10.1016/j.ejmech.2015.04.040.

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D., Bitto, A., 2017. Oxidative Stress: Harms and Benefits for Human Health. Oxid. Med. Cell Longev.; 2017:8416763. DOI: 10.1155/2017/8416763.

Poléo, A.B.S., Schjolden, J., Sørensen, J., Nilsson, G.E., 2017. The high tolerance to aluminium in crucian carp (Carassius carassius) is associated with its ability to avoid hypoxia. PLoS One; 12(6):e0179519. DOI: 10.1371/journal.pone.0179519.

Poljsak, B., Šuput, D., Milisav, I., 2013. Achieving the Balance between ROS and Antioxidants: When to Use the Synthetic Antioxidants, Oxid. Med. Cell Longev.; 956792: 11, https://doi.org/10.1155/2013/956792.

Praskova, E., Plhalova, L., Chromcova, L., Stepanova, S., Bedanova, I., Blahova, J., Hostovsky, M., Skoric, M., Maršálek, P., Voslarova, E., Svobodova, Z., 2014. Effects of subchronic exposure of diclofenac on growth, histopathological changes, and oxidative stress in zebrafish (Danio rerio). ScientificWorldJournal; 2014:645737. DOI: 10.1155/2014/645737.

Rock, K.D., Patisaul, H.B., 2018. Environmental Mechanisms of Neurodevelopmental Toxicity. Curr. Environ. Health Rep.; 5(1):145-157. DOI: 10.1007/s40572-018-0185-0.

Santos, D., Luzio, A., Coimbra, A.M., 2017. Zebrafish sex differentiation and gonad development: A review on the impact of environmental factors. Aquat. Toxicol.; 191,141–163. DOI: 10.1016/j.aquatox.2017.08.005.

Sayed, A.E.D.H., Mekkawy, I.A., Mahmoud, U.M. and Nagiub, M., 2020. Histopathological and histochemical effects of silver nanoparticles on the gills and muscles of African catfish (Clarias garepinus). Scientific African, 7, e00230. https://doi.org/10.1016/j.sciaf.2019.e00230.

Senger, M.R., Seibt, K.J., Cordenonzi, G., Dutra Dias, R., Bogo, M.R., Bonan, C.D., 2011. Aluminum exposure alters behavioral parameters and increases acetylcholinesterase activity in zebrafish (Danio rerio) brain. Cell Biol. Toxicol.; 27(3), 199-205. DOI: 10.1007/s10565-011-9181-y.

Sharma, S., Wakode, S., Sharma, A., Nair, N., Dhobi, M., Wani, M.A., Pottoo, F.H., 2020. Effect of environmental toxicants on neuronal functions. Environ. Sci. Pollut. Res. Int. ; 27(36):44906-44921. DOI: 10.1007/s11356-020-10950-6.

Shi, H., Sui, Y., Wang, X., Luo, Y., Ji, L., 2005. Hydroxyl radical production and oxidative damage induced by cadmium and naphthalene in liver of Carassius auratus. Comp. Biochem. Physiol. C Toxicol. Pharmacol.; 140(1):115-21. DOI: 10.1016/j.cca.2005.01.009.

Silva Santos, N., Oliveira, R., Lisboa, C.A, Pinto, J.M.E, Sousa-Moura, D., Camargo, N.S., Perillo, V., Oliveira, M., Grisolia, C.K., Domingues, I., 2018. Chronic effects of carbamazepine on zebrafish: Behavioral, reproductive and biochemical endpoints. Ecotoxicol. Environ. Saf.; 164, 297–304. DOI:10.1016/j.ecoenv.2018.08.01.

Singla, N., Dhawan, D.K., 2014. Zinc modulates aluminium-induced oxidative stress and cellular injury in rat brain. Metallomics; 6(10),1941–1950. DOI:10.1039/c4mt00097h.

Smuder, A.J., Kavazis, A.N., Hudson, M.B., Nelson, W.B., Powers, S.K., 2010. Oxidation enhances myofibrillar protein degradation via calpain and caspase-3. Free Radic. Biol. Med.; 49(7):1152-60. DOI: 10.1016/j.freeradbiomed.2010.06.025.

Souza, J.P., Mansano, A.S., Venturini, F.P., Santos, F., Zucolotto, V., 2019. Antioxidant metabolism of zebrafish after sub-lethal exposure to graphene oxide and recovery. Fish Physiol. Biochem.; 45, 1289–1297. https://doi.org/10.1007/s10695-019-00678-7.

Valvona, C.J., Fillmore, H.L., Nunn, P.B., Pilkington, G.J., 2016. The Regulation and Function of Lactate Dehydrogenase A: Therapeutic Potential in Brain Tumor. Brain Pathol.; 26(1):3-17. DOI: 10.1111/bpa.12299.

Westerfield, M. (2000). The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), fourth ed. University of Oregon Press, Eugene.

WHO, Geneva, Switzerland, 1997. Environmental Health Criteria for Aluminium. Environmental Health Criteria No.194. pp.xx + 282 pp. ref.many. ISSN: 0250-863X.

WHO, Guidelines for Drinking-water Quality, 2013. Aluminium in drinking-water. WHO/HSE/WSH/10.01/13.

Wu, M.P., Chang, N.C., Chung, C.L., Chiu, W.C., Hsu, C.C., Chen, H.M., Sheu, J.R., Jayakumar, T., Chou, D.S., Fong, T.H., 2018. Analysis of titin in red and white muscles: crucial role on muscle contractions using a fish model.Biomed. Res. Int., 5816875:11. https://doi.org/10.1155/2018/5816875.

Yang, M., Qiu, W., Chen, J., Zhan, J., Pan, C., Lei, X., Wu, M., 2014. Growth inhibition and coordinated physiological regulation of zebrafish (Danio rerio) embryos upon sublethal exposure to antidepressant amitriptyline. Aquat. Toxicol.; 151:68-76. DOI: 10.1016/j.aquatox.2013.12.029.

Yokel, R.A., 2020. Aluminum reproductive toxicity: a summary and interpretation of scientific reports. Crit. Rev. Toxicol.; 50(7):551-593. DOI: 10.1080/10408444.2020.1801575.

Preliminary study of histological changed and oxidative stress of zebrafish gills after aluminium exposure

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Abstract

Gills are key organs to assess aquatic contamination in fish due to their direct and permanent contact with contaminants in the water. Aluminium is considered a dangerous pollutant especially for aquatic organisms, since the phenomenon of acid rain makes the metal more available in water bodies. In light of this phenomenon, the aim of this study is to obtain preliminary data of aluminium effects on the gills of fish, since these represent the first organ interacting with the metal once dissolved in water. Zebrafish has been chosen as an experimental model, since it is reported as a bioindicator for the evaluation of ecotoxicological effects. The adult specimens were exposed at a concentration of 11 mg/L for 10, 15 and 20 days and the effects on both branchial morphology and oxidative stress induced were analysed. Specifically, ROS content, antioxidant enzymes activity, oxidative damage to lipids and *in vitro* susceptibility to oxidative stress were evaluated. The results indicate that aluminium acts by altering both tissue histology and oxidative state, increasing ROS levels, lipid hydroperoxides, and the activity of antioxidant enzymes such as glutathione peroxidase and glutathione reductase. However, the antioxidant system is more efficient after 20 days of exposure suggesting an adaptive mechanism that makes the organisms less susceptible to aluminium induced oxidative stress.

Overall, the results provide an important starting point for assessing the toxicity induced by aluminium on the gills of aquatic organisms, the effects of which are still poorly understood.

Keywords: Antioxidant enzymes, ecotoxicology, *Danio rerio*, lipid hydroperoxides, oxidative damage, ROS.

1. Introduction

Gills represent the first tissue to get into contact with several kinds of pollutants in water bodies. They are multifunctional organs involved in critical physiological processes, including the exchange of O_2 and CO_2, and the control of respiration by peripheral chemoreceptors (Evans et al., 2005). Being involved in gaseous exchange, gills tissue is metabolically active and it can accumulate a significant proportion of toxins present in water bodies (Strzyzewska et al., 2016). Therefore, the gills are considered biomarkers of aquatic pollution, and their pollutants content has been found similar to the concentration found in the environment inhabited by fishes (Strzyżewska-Worotyńska et al., 2017). Furthermore, the gills serve as early warning signs on the health status of fish (Sorour, 2001), since tissue alterations or functional abnormalities appear earlier in these organs rather than in others (Heier et al. 2009; Yancheva et al. 2015). Aluminium (Al) is considered a dangerous pollutant especially for the aquatic compartment (Tchounwou et al., 2012), as its concentration inside it can reach very high levels, up to 50 mg/L (ATSDR, 2008) compared to the values considered normal (0.001-0.05 mg/L) (WHO, 1997, 2013). Acid rainfalls play a crucial role in the Al bio-availability, inducing its mobilization from bottom sediments and increasing its concentration in water bodies (Lawrence et al., 2007). Thus, Al becomes more soluble and potentially more toxic to aquatic biota at acidic pH (Gensemer and Playle, 1999). Studies carried out with different fish species have revealed that Al can produce toxic effects in fish by disturbing physiological activities (Allin and Wilson, 2000), biochemical processes (Poleo and Hytterod, 2003), fertility (Keinanen et al., 2003), growth (Vuorinen et al., 2003), and mortality (Teien et al., 2005). The toxicity mechanisms of Al exposure are poorly understood and it has been reported that the metal can potentiate the pro-oxidant effects of Fe and Cu, which are present in most cell compartments, so increasing reactive oxygen species (ROS) (Kumar and Gill, 2009). When ROS production overwhelm antioxidant system activity, a condition

of oxidative stress onset (Sies et al., 2017). In our previous experiments we showed alterations in swimming activity of *Danio rerio* (Capriello et al., 2019, 2021a) and changes in the oxidative state of brain in the same experimental model (Capriello et al., 2021a). To date, scarce information has been reported about Al toxicity mechanisms on gills tissue. In our study, *Danio rerio* (zebrafish) was chosen as model organism being considered very useful for studying the ecotoxicological effects of various pollutants (Favorito et al., 2011). The animals were exposed to a concentration of 11 mg/L Al using $AlCl_3 \cdot 6H_2O$ as a source of Al and the effects on morphology and oxidative state of the gills were analysed after 10, 15 and 20 days of exposure. The concentration, used also in our previous experiments (Capriello et al., 2021a), simulates the conditions found in polluted surface waters (Agarwal et al., 2016, ATSDR, 2008) and takes into account the Al dose-lethality curve evaluated in one previous study (Monaco et al., 2017). In this study we evaluated histology of gills tissue, ROS content, oxidative damage to lipids, antioxidant enzymes activities, namely glutathione reductase (GR) and glutathione peroxidase (GPx), and *in vitro* susceptibility to oxidative stress.

Overall, this research aims to study the toxicity induced by Al on aquatic organisms, adding information of the dangerousness of this pollutant.

2. Materials and Methods

2.1 Zebrafish breeding

Adult zebrafish were housed in tanks under 12 h light:12 h dark photoperiod, at a temperature of 28 °C, and fed twice a day with a commercial diet supplemented with *Artemia* sp. nauplii (Westerfield, 2000). They were lodged at the Department of Biology of the University of Naples “Federico II” and all used protocols were in accordance with the guidelines dictated by European regulations on animal of welfare (Directive 2010/63/EU) and approved by the Italian Ministry of Health (permits 147/2019-PR).

2.2 Al exposure and samples collection

The treatment solution of 11 mg/L of Al, was prepared by dissolving 2.5 g of $AlCl_3 \cdot 6H_2O$ (Carlo Erba, Italy; CAS no. 7784-13-6), in 25 L (0.1 g/L) of breeding solution directly (1/3 bidistilled water, 2/3 tap water) in the tank. Three groups with each 8 healthy zebrafish, between 6-12 months of life, were placed in a 25 L tanks and exposed respectively for 10, 15 and 20 days to 0.1 g/L of $AlCl_3 \cdot H_2O$ solution (11 mg/L Al). Another group of 8 fish, exposed only to tank water (1/3 bidistilled water, 2/3 tap water), was used as a control group. A biological triplicate was performed for each treatment. Five gills, from the replicates of each experimental group, were used to conduct the biochemical analyses in order to evaluate the oxidative state. Similarly three gills for each experimental group from each replicate, were collected and prepared for histological analysis. All fish were sacrificed by an overdose of MS-222 solution (300 mg/L).

2.3 Histological analyses

Standard histological protocols for optical microscopy were peroformed for all gills. The samples were fixed in Bouin solution for 48 h to preserve the protoplasmic structure, dehydrated and included in paraffin. Sagittal sections of 5 μm, deparaffinized and hydrated, were stained by trichrome of Mallory (Capriello et al., 2021b). At the end, all images were acquired with a Kontron Electronic Imaging System KS300 (Zeiss, Germany).

2.4 Oxidative stress analyses

Six gills from each treatment group were homogenized into 1 mL of ice-cold homogenization medium (HM) (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4) using a glass Potter-Elvehjem homogenizer set at a standard velocity (500 rpm) for 1 min. Protein concentration in homogenates was measured by the biuret method (Gornall et al., 1949). Homogenate aliquots were used to measure ROS levels, oxidative damage, *in vitro* susceptibility to oxidative stress and antioxidant enzyme activities.

2.4.1 Tissue ROS content

The ROS content was measured following the ROS-induced conversion of 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA, nonfluorescent compound in dichlorofluorescein (DCF, fluorescent compound) according to Driver et al., (2000). In brief, 12.5 µg of homogenate proteins in 200 µL of monobasic phosphate buffer 0.1 M, pH 7.4, were incubated for 15 minutes with 10 µM DCFH-DA. Then, 100 µM $FeCl_3$ was added, and the mixture was incubated for 30 minutes. The conversion of DCFH-DA to the fluorescent product DCF was measured using a multimode microplate reader (Synergy™ HTX Multimode Microplate Reader, BioTek) with excitation and emission wavelengths of 485 and 530 nm, respectively. Background fluorescence (conversion of DCFH to DCF in the absence of homogenate and mitochondria) was corrected with parallel blanks.

2.4.2 Oxidative damage and in vitro susceptibility to oxidant

The level of lipid hydroperoxides (HP) were used to measure the extent of the lipid peroxidative processes in homogenates according to Heath and Tappel (Heath and Tappel, 1976). Susceptibility to oxidative stress of gills tissue was evaluated by the change in hydroperoxide levels following the treatment of 10 % tissue homogenate with iron and ascorbate (Fe/As), at a concentration of 100/1000 µM, for 10 min at room temperature. The reaction was blocked by adding 0.2% 2, 6-di-t-butyl-p-cresol (BHT) and the hydroperoxide levels were evaluated as previously described.

2.4.3 Antioxidant enzyme activity

GPx activity of 0.01 mg tissue homogenate proteins was assayed at 30°C, with H_2O_2 as substrate, in the presence of GSH (reduced glutathione), (Flohé and Günzler, 1984) following the rate of NADPH oxidation, necessary to reduce GSSG (oxidized glutathione), and catalysed by adding GR.

GR homogenate activity of 0.01 mg tissue homogenate proteins was assayed at 30°C, measuring the rate of NADPH oxidation of following the addition of GSSG (Carlberg and Mannervik, 1975). For each procedure, the rate of NADPH oxidation was followed at 340 nm using a multi-mode microplate reader (Synergy™ HTX Multi-Mode Microplate Reader, BioTek).

2.5 Statistical analysis

Statistically significant differences among treatments were assessed by Graph Pad Prism 8 (GraphPad Software, La Jolla California USA, www.graphpad.com), using one-way ANOVA followed by Tukey's pairwise comparison tests. Data were expressed as mean ± SEM and were considered to be statistically significant at $P < 0.05$.

3. Results

3.1 Morphology of gills

The controls gill apparatus presented a homogeneous tissue with numerous and ordered primary and secondary lamellae defined (Fig 1A) separated by large spaces (Fig 1B) or joined by epithelial tissue (Fig. 1C). All the organisms treated showed alterations in the morphology of the brachial tissue albeit with different degrees of alterations. The gills of organisms exposed to Al at 10 days showed highly damaged branchial tissue with damaged primary lamellae (Fig 1D), few secondary lamellae separated by little space and lacking epithelial tissue (Fig.1E) often irregular, short and curl (Fig. 1F).

In organisms exposed to 15 days, the branchial tissue still showed other alterations, although it was more orderly and with more numerous lamellae (Fig. 1G), the secondary lamellae still had an irregular shape (Fig. 1H) with the presence of aneurysms (Fig 1I).

In organisms exposed to Al at 20 days, the tissue appeared to have less altered morphology than organisms exposed to less time to treatment. The lamellae were more ordered and numerous (Fig. 1J) and the quantity of lamellae with irregular morphology was decreased (Fig. 1K). However, some

secondary lamellae appeared fused and indistinguishable from each other, with little space to separate them (Fig. 1L).

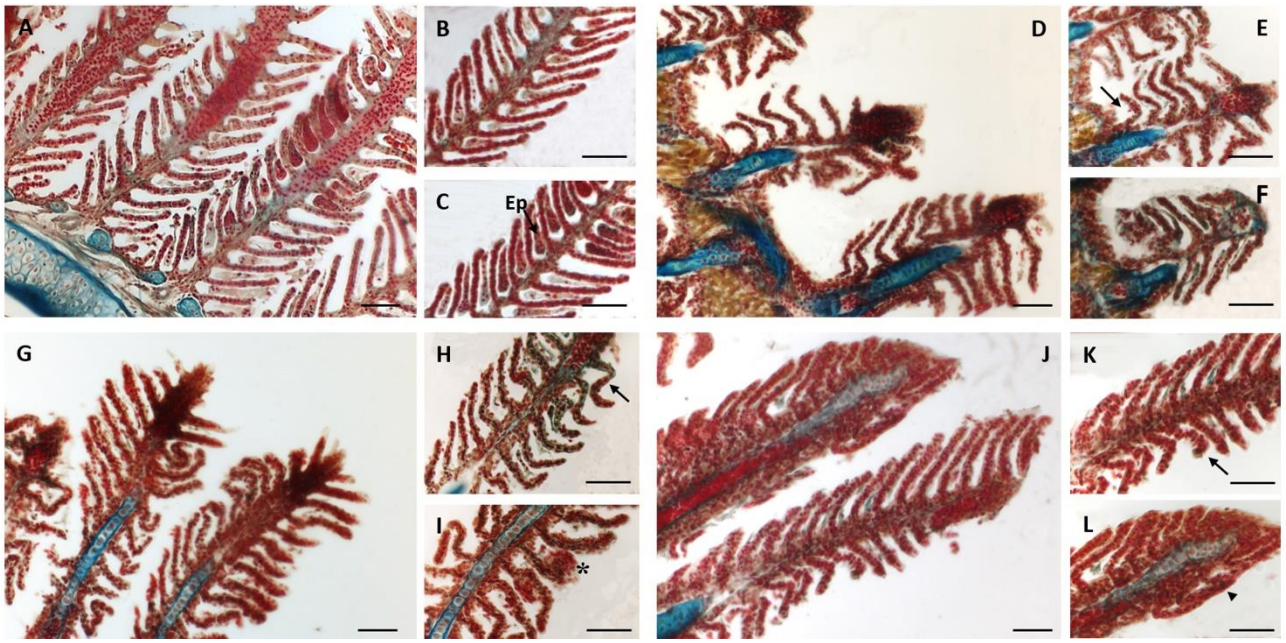

Fig. 1 Trichrome of Mallory staining of zebrafish gills. (A, B, C) Control gills. (D, E, F) Gills of organisms exposed to Al for 10 days. (G, H, I) Gills of organisms exposed to Al for 15 days. (J, K, L) Gills of organisms exposed to Al for 20 days. The control tissue appears homogeneous and ordered with secondary lamellae separated by spaces and epithelial tissue (Ep). In the treated organisms the tissue was altered with secondary lamellae irregular and bent (arrows ➙). In particular, organisms exposed to Al for 15 days showed also aneurysms (*) and those exposed to 20 days fused secondary lamellae (arrow ▼). Scale bars: 50 μm.

3.2 ROS content and lipid hydroperoxides

The levels of ROS are significantly increased in gills of all organisms exposed to Al. Organisms exposed to 10 and 15 days have a significant increase ($p < 0.0001$) in the amount of ROS compared to controls. This accumulation was further increased for the maximum exposure time $p < 0.0001$. Instead, the lipid hydroperoxides level (HPs) increased significantly in organisms exposed for 10 days of Al-treatment ($p < 0.0001$) compared to those of control, and increased even more in homogenates of gills exposed to Al for 15 days ($p < 0.0001$). However, after 20 days of exposure, the level of HPs significantly decreases than ones revealed after 15 days of treatment ($p < 0.0001$), although the values

remained higher than those found in controls and organisms exposed to 10 days of Al-treatment.

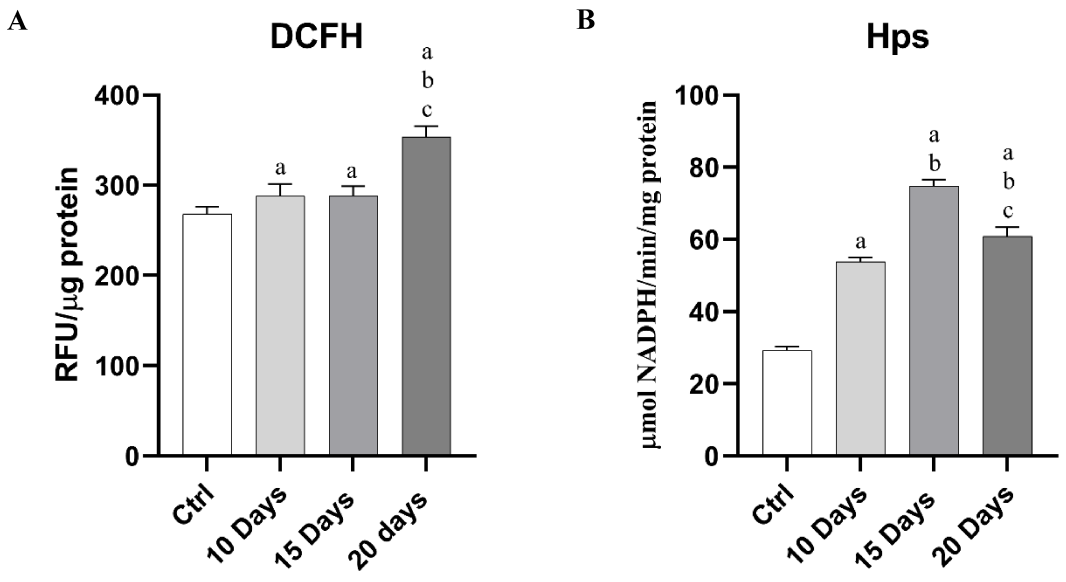

Fig. 2 (A) The graph shows ROS content in gills zebrafish homogenates. The levels of ROS are significantly increased in gills of all organisms exposed to Al. The increase seems to be time-dependent with values highest at 20 exposition days. (B) In the graph it is reported the lipid hydroperoxides level. The amount of HPs is increased after 10 days of treatment and even more in homogenates of gills exposed to Al for 15 days. The values detected after 20 days of exposition are significantly lower than ones revealed after 15 days of treatment, but significantly higher respect the values of HPs detected in the control and in organisms exposed to Al for 10 days. Different lowercase letters indicate significant differences between groups.

3.3 Activity of antioxidant enzymes and susceptibility to oxidative stress

The activity of antioxidant enzymes was altered by treatment with Al. Specifically, the activity of GPx was significantly increased in the gills of all treated organisms ($p < 0.0001$) compared to control and tended to remain constant for all treatment periods.

The activity of the GR enzyme was also increased for Al-treated organisms compared to controls, already after short exposures (10 days, $p < 0.0001$) but increased further after longer exposure times (15 and 20 days, $p < 0.0001$).

A significant increase in susceptibility to oxidative stress, evaluated *in vitro*, was recorded in the gill homogenates of organisms exposed for 10 and 15 days to treatment ($p < 0.0001$). However, this value

decreased after 20 days of Al exposure ($p < 0.0001$) compared to shorter exposure times, although it remained higher than that found in the controls.

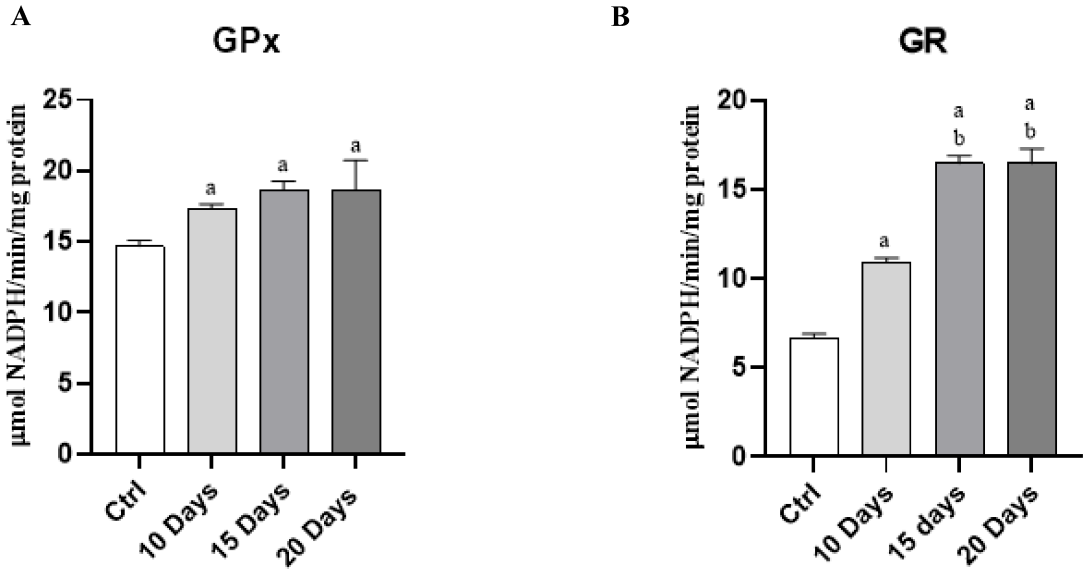

Fig. 3 The graphs show antioxidant enzymes activity in gills homogenates. (A) GPx activity is significantly increased in all exposed groups respect to the control one. (B) The GR activity increases after Al exposure, with highest values after 15 and 20 days of treatment. Different lowercase letters indicate significant differences between groups.

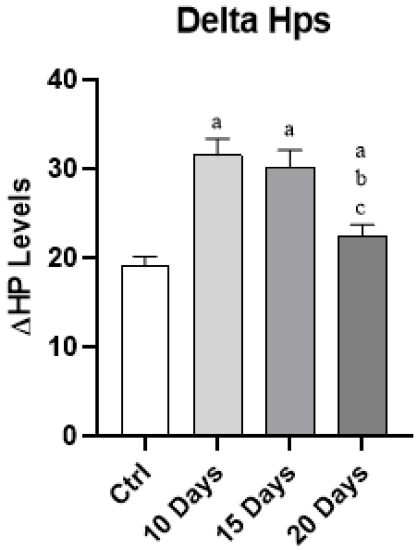

Fig. 4 In the graph it is reported the *in vitro* susceptibility to oxidative stress. The homogenates samples exposed to 10 and 15 days of Al-treatment are significantly more susceptible to an *in vitro* oxidative stress respect to the control group. The exposition for 20 days significantly reduces the susceptibility respect to the organisms exposed to Al for 10 and 15 days. Different lowercase letters indicate significant differences between groups.

4. Discussion

Our results highlight for the first time that Al dissolved in water can alter the morphology and redox state of zebrafish gills. The gills are the first point of contact with environmental pollutants in the water (Strzyzewska et al., 2016). They are multifunctional organs, handling vital functions such as respiration, osmoregulation, acid-base balance and excretion of nitrogenous residues (Evans et al., 2005). Paradoxically, they are highly vulnerable to toxic chemicals primarily because of their large surface area which facilitates increased toxic interaction and absorption, and also because the gill detoxification system is not as robust as that of other organs (Playle and Wood, 1990; Pereira et al., 2010). Thus, the gills are often used in assessing the impact of water pollutants on aquatic habitats, providing important indications regarding their toxicity (Atli et al., 2006) and reliable results for assessing environmental water contamination (Pereira et al., 2010). Several studies reported that Al can be extremely toxic to fish affecting the function of fish gills, and the main consequences are respiratory and ion regulatory dysfunctions (Howells et al., 1990; Neville, 1985). Furthermore, in our previous studies it has already shown that Al can alter the swimming activity and oxidative state of zebrafish larvae (Capriello et al., 2019) but also induce behavioural changes and neurotoxicity in adults of zebrafish (Capriello et al., 2021a).

The histopathological alterations found in this study such as shortening and curling of secondary lamellae, aneurisms or lamellar fusion were reported for a wide variety of aquatic xenobiotics, e.g. metals, pharmaceuticals, pesticides (Ahmed et al., 2013; Pal et al., 2012; Rodrigues et al., 2017). In this case the alterations were more evident after short periods. Indeed, it is known that alterations in gill morphology usually appear immediately after acute exposure to pollutants, because reflected the direct effect of toxicants (Mallatt, 1985). However, at longer exposure times, while presenting a tissue that apparently comes closest to that of the control organisms, alterations of a different nature were still present such as aneurysms or lamellar fusion. In the literature, it is reported that the aneurysms with the consequent loss of vascular integrity occur due to an increase of blood in the lamellae (Ahmed et al., 2013; Pal et al., 2012), while lamellar fusion and consequent reduction in gas exchange

area occurs in an attempt to create a barrier to the entry of pollutants (Cengiz, 2006; Monteiro et al., 2008). Both of these mechanisms, which also occurred in the gills of various aquatic organisms, after long or high exposures of other pollutants (Cengiz, 2006; Monteiro et al., 2008; Pal et al., 2012; Rodrigues et al., 2017) can be interpreted as an adaptation defense mechanism in fish, per hinder the access of toxicants to the body. This hypothesis was also confirmed by the trend of the oxidative state of the gills analysed at the different times of Al exposure.

In particular, ROS levels are increased during all three times of treatment with highest levels after 20 days of exposure. These data are supported by literature reporting which Al toxicity is both dose- and time- dependent and significantly correlated with ROS production (Chandra et al., 2020). Despite numerous reports on the involvement of oxidative stress in Al toxicity (Chowra et al., 2017; Ma et al., 2007), it should be noted that since Al itself is not a transition metal, it couldn't catalyse redox reactions. However, it is known that this metal can act as a pro-oxidant in both *in vitro* and *in vivo* situations (Exley, 2004; Yoshino et al., 1999). The exact mechanism for its pro-oxidant activity is still the subject of investigation but it is known that Al has a strong affinity for bio-membranes and causes their rigidification (Wu et al., 2014), facilitating free radical chain reactions [mediated by Ferrous (Fe) ions] which promote the peroxidation of membrane lipids. In according to this mechanism, in our experiments we demonstrate that hydroperoxides levels enhance during Al treatment. The increase seems to be time-dependent until 15 days of exposure and reflects the increase of ROS content after 10 and 15 days of treatment. After 20 days of Al exposure, Hps level undergoes a reduction notwithstanding the significant increase of ROS content. It is possible hypothesize that this can due to a reinforcement of antioxidant defense system. Indeed, the GPx and GR activities are increased by Al exposition, and the GR activity is more raised after 15 and 20 days of treatment.

Indeed, after 20 days of treatment, a decrease in susceptibility to stress is also evident, also confirmed by a better morphology of the branchial apparatus. This stabilization/improvement of conditions, which has already occurred in zebrafish after exposure to other pollutants (Acosta et al., 2016; Avallone et al., 2015; Pilehvar et al., 2020), and also highlighted in the brain exposed to the same

conditions (Capriello et al., 2021a), is linked to the zebrafish's ability to activate an efficient detoxifying system that allows it to survive even in stressful environments.

In conclusion, the results relating to the functional and histological changes detected in zebrafish gills, highlight the toxicity mechanisms of the metal on aquatic organisms and invite reflection on the danger deriving from its growing diffusion. Further studies are required to understand the involvement of cell ROS producers such as mitochondria and NADPH oxidase.

Conflict of interest

The authors declare that they have no financial interests or personal relationships that could influence the content of the paper

References

Acosta, D.D.S., Danielle, N.M., Altenhofen, S., Luzardo, M.D., Costa, P.G., Bianchini, A., Bonan, C.D., da Silva, R.S., Dafre, A.L., 2016. Copper at low levels impairs memory of adult zebrafish (Danio rerio) and affects swimming performance of larvae. Comp. Biochem. Physiol. C Toxicol. Pharmacol.; 185-186:122-130. DOI: 10.1016/j.cbpc.2016.03.008.

Agarwal, S., Tyagi, I., Gupta,V.K., Dehghani, M.H., Ghanbari, R., 2016. Investigating the residual aluminum elimination from conventional and enhanced coagulation by phosphate compounds in wastewater treatment process. J. Mol. Liq.; 221:673-684.https://doi.org/10.1016/j.molliq.2016.06.051.

Agency for Toxic Substances and Disease (ATSDR), 2008. Toxicological Profile for Aluminum. CAS: 7429-90-5.

Ahmed, M.K., Habibullah-Al-Mamun, M., Parvin, E., Akter, M.S., Khan, M.S., 2013. Arsenic induced toxicity and histopathological changes in gill and liver tissue of freshwater fish, tilapia (Oreochromis mossambicus). Exp. Toxicol. Pathol.; 65(6):903-9. DOI: 10.1016/j.etp.2013.01.003.

Allin, C.J., Wilson, R.W., 2000. Effects of pre-acclimation to aluminium on the physiology and swimming behaviour of juvenile rainbow trout Oncorhynchus mykiss during a pulsed exposure. Aquatic Toxicology, 51(2), 213-224. DOI: 10.1016/s0166-445x(00)00111-9.

Atli, G., Alptekin, O., Tükel, S., Canli, M., 2006. Response of catalase activity to Ag+, Cd2+, Cr6+, Cu2+ and Zn2+ in five tissues of freshwater fish Oreochromis niloticus. Comp. Biochem. Physiol. C Toxicol. Pharmacol.; 143(2):218-24. DOI: 10.1016/j.cbpc.2006.02.003.

Avallone, B., Agnisola, C., Cerciello, R., Panzuto, R., Simoniello, P., Cretì, P., Motta, C.M., 2015. Structural and functional changes in the zebrafish (Danio rerio) skeletal muscle after cadmium exposure. Cell Biol. Toxicol.; 31(6):273-83. DOI: 10.1007/s10565-015-9310-0.

Carlberg, I., Mannervik, B., 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J Biol. Chem.; 250(14):5475-80.

Capriello, T., Félix, L.M., Monteiro, S.M., Santos, D., Cofone, R., Ferrandino, I., 2021a. Exposure to aluminium causes behavioural alterations and oxidative stress in the brain of adult zebrafish. Environ. Toxicol. Pharmacol.; 85:103636. DOI: 10.1016/j.etap.2021.103636.

Capriello, T., Grimaldi, M.C., Cofone, R., D'Aniello, S., Ferrandino, I., 2019. Effects of aluminium and cadmium on hatching and swimming ability in developing zebrafish. Chemosphere; 222:243-249. DOI: 10.1016/j.chemosphere.2019.01.140.

Capriello, T., Visone, I.M., Motta, C.M., Ferrandino, I., 2021b. Adverse effects of E150d on zebrafish development. Food Chem. Toxicol; 147:111877. DOI: 10.1016/j.fct.2020.111877.

Cengiz, E.I., 2006. Gill and kidney histopathology in the freshwater fish Cyprinus carpio after acute exposure to deltamethrin. Environ. Toxicol. Pharmacol.; 22(2):200-4. DOI: 10.1016/j.etap.2006.03.006.

Chandra, J., Parkhey, S., Varghese, D., Varghese, B. and Keshavkant, S., 2020. Aluminium Rhizotoxicity in Cicer arietinum. *Russian Journal of Plant Physiology*; *67*(5):945-952. https://doi.org/10.1134/S1021443720050027.

Chowra, U., Yanase, E., Koyama, H., Panda, S.K., 2017. Aluminium-induced excessive ROS causes cellular damage and metabolic shifts in black gram Vigna mungo (L.) Hepper. Protoplasma.; 254(1):293-302. DOI: 10.1007/s00709-016-0943-5.

Driver, A.S., Kodavanti, P.R., Mundy, W.R., 2000. Age-related changes in reactive oxygen species production in rat brain homogenates. Neurotoxicol. Teratol.; 22(2):175-81. DOI: 10.1016/s0892-0362(99)00069-0.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev.; 85(1):97-177. DOI: 10.1152/physrev.00050.2003.

Exley, C., 2004. The pro-oxidant activity of aluminum. Free Radic. Biol. Med. 36 (3), 380–387. https://doi.org/10.1016/j.freeradbiomed.2003.11.017

Favorito, R., Chiarelli, G., Grimaldi, M.C., De Bonis, S., Lancieri, M., Ferrandino, I., 2011. Bioaccumulation of cadmium and its cytotoxic effect on zebrafish brain. Chem. Ecol.; 27(2),39-46. https://doi.org/10.1080/02757540.2011.625937.

Flohé, L., Günzler, W.A., 1984. Assays of glutathione peroxidase. Methods Enzymol.; 105:114–120.

Gensemer, R.W., Playle, R.C., 1999). The bioavailability and toxicity of aluminium in aquatic environments. Crit. Rev. Environ. Sci. Technol., 29, 315-450 *https://doi.org/10.1080/10643389991259245.*

Gornall, A.G.; Bardawill, C.J.; David, M.M., 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem.; 177:751-766.

Heath, R.L.; Tappel, A.L., 1976. A new sensitive assay for the measurement of hydroperoxides. Anal Biochem.; 76:184-191.

Heier, L.S., Lien, I.B., Strømseng, A.E., Ljønes, M., Rosseland, B.O., Tollefsen, K.E., Salbu, B., 2009. Speciation of lead, copper, zinc and antimony in water draining a shooting range--time dependant metal accumulation and biomarker responses in brown trout (Salmo trutta L.). Sci. Total Environ.; 407(13):4047-55. DOI: 10.1016/j.scitotenv.2009.03.002.

Howells, G., Dalziel, T.R.K., Reader, J.P., Solbe, J.F., 1990. Eifac Water Quality Criteria for European Freshwater Fish: Report On Aluminium, Chemistry and Ecology, 4:3, 117-173, DOI: 10.1080/02757549008035972.

Keinanen, M., Tigerstedt, C., Kalax. P., Vuorinen, P.J. (2003). Fertilization and embryonic development of whitefish Coregonus lavaretus lavaretus in acidic low-ionic-strength water with aluminum. Ecotoxicol. Environ. Sa., 55(3), 314-29

Kumar V, Gill KD. Aluminium neurotoxicity: neurobehavioural and oxidative aspects. Archives of Toxicology. 2009 Nov;83(11):965-978. DOI: 10.1007/s00204-009-0455-6.

Lawrence, G.B., Sutherland, J.W., Boylen, C.W., Nierzwicki-Bauer, S.W., Momen, B., Baldigo, B.P., Simonin, H.A., 2007. Acid rain effects on aluminum mobilization clarified by inclusion of strong organic acids. Environ. Sci. Technol. 41 (1), 93–98. https://doi.org/10.1021/es061437v.

Ma, B., Wan, J. and Shen, Z., 2007. H_2O_2 production and antioxidant responses in seeds and early seedlings of two different rice varieties exposed to aluminum. *Plant Growth Regulation*; *52*(1):91-100. https://doi.org/10.1007/s10725-007-9183-1

Mallat, J., 1985. Fish gill structural changes induced by toxicants and other irritants: a statistical review. Can. J. Fish. Aquat. Sci. 42, 630–648. DOI: 10.1139/f85-083.

Monaco, A., Grimaldi, M.C., Ferrandino, I., 2017. Aluminium chloride-induced toxicity in zebrafish larvae. J. Fish. Dis.; 40(5), 629-35. DOI: 10.1111/jfd.12544.

Monteiro, S.M., Rocha, E., Fontaínhas-Fernandes, A., Sousa, M., 2008. Quantitative histopathology of Oreochromis niloticus gills after copper exposure. J. Fish Biol. 73:1376–1392. https://doi.org/10.1111/j.1095-8649.2008.02009.x.

Neville, C.M., Campbell, P.G.C., 1988. Possible mechanisms of aluminum toxicity in a dilute, acidic environment to fingerlings and older life stages of salmonids. Water Air Soil Pollut .; 42:311–327.

Pal, S., Kokushi, E., Koyama, J., Uno, S., Ghosh, A.R., 2012. Histopathological alterations in gill, liver and kidney of common carp exposed to chlorpyrifos. J. Environ. Sci Health B.; 47(3):180-95. DOI: 10.1080/03601234.2012.632285.

Pereira, P., de Pablo, H., Vale, C., Pacheco, M., 2010. Combined use of environmental data and biomarkers in fish (Liza aurata) inhabiting a eutrophic and metal-contaminated coastal system - Gills reflect environmental contamination. Mar Environ Res.; 69(2):53-62. DOI: 10.1016/j.marenvres.2009.08.003.

Pilehvar, A., Town, R.M., Blust, R., 2020. The effect of copper on behaviour, memory, and associative learning ability of zebrafish (Danio rerio). Ecotoxicol. Environ. Saf.; 188-109900. DOI: 10.1016/j.ecoenv.2019.109900.

Playle, R.C., Wood, C.M.,1990. Is precipitation of aluminum fast enough to explain aluminum deposition on fish gills? Can. J. Fish. Aquat. Sci., 47:1558-1561.

Poleo, A., Hytterød, S..,2003. The effect of aluminium in Atlantic salmon Salmo salar with special emphasis on alkaline water. Journal of Inorganic Biochemistry, 97, 89–96.

Rodrigues, S., Antunes, S.C., Nunes, B., Correia, A.T., 2017. Histological alterations in gills and liver of rainbow trout (Oncorhynchus mykiss) after exposure to the antibiotic oxytetracycline. Environ. Toxicol. Pharmacol.; 53:164-176. DOI: 10.1016/j.etap.2017.05.012.

Sies H, Berndt C, Jones DP., 2017. Oxidative Stress. Annu Rev Biochem.;;86:715-748. DOI: 10.1146/annurev-biochem-061516-045037.

Sorour, J., 2001. Ultrastructural variations in Lethocerus niloticum (Insecta: Hemiptera) caused by pollution in Lake Mariut, Alexandria, Egypt. Ecotoxicol. Environ. Saf. ; 48(3):268-74. DOI: 10.1006/eesa.2000.2003.

Strzyzewska, E., Szarek, J., Babinska, I., 2016. Morphologic evaluation of the gills as a tool in the diagnostics of pathological conditions in fish and pollution in the aquatic environment: a review. Veterinarni Medicina, 61:123-132. https://doi.org/10.17221/8763-VETMED.

Strzyżewska-Worotyńska, E., Szarek, J., Babińska, I., Gulda, D., 2017. Gills as morphological biomarkers in extensive and intensive rainbow trout (Oncorhynchus mykiss, Walbaum 1792) production technologies. Environ. Monit .Assess.; 189(12):611. DOI: 10.1007/s10661-017-6278-7.

Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy metals toxicity and the environment. EXS 101, 133–164. https://doi.org/10.1007/978-3-7643-8340-4_6

Teien, H.C., Salbu, B., Heier ,L.S., Kroglund ,F., Rosseland, B.O., (2005). Fish mortality during sea salt episodes—catchment liming as a countermeasure. J Environ Monit., 7(10), 989-998.

Vuorinen, P.J., Keinanen, M., Peuranen, S., Tigerstedt, C., 2003. Reproduction, blood and plasma parameters and gill histology of vendace Coregonus albula L. in long-term exposure to acidity and aluminum. Ecotoxicol Environ Saf., 54(3), 255-276. DOI: 10.1016/s0147-6513(02)00078-7.

Westerfield, M., 2000. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), fourth ed. University of Oregon Press, Eugene.

WHO Guidelines for Drinking-water Quality, 2013. Aluminium in drinking-water. WHO/ HSE/WSH/10.01/13.

World Health Organization, Geneva, Switzerland, 1997. Environmental Health Criteria for Aluminium. Environmental Health Criteria No.194. pp.xx + 282 pp. ref.many. ISSN: 0250-863X.

Wu, D., Shen, H., Yokawa, K. and Baluška, F., 2014. Alleviation of aluminium-induced cell rigidity by overexpression of OsPIN2 in rice roots. *Journal of Experimental Botany*, *65*(18): 5305-5315. https://doi.org/10.1093/jxb/eru292.

Yancheva, V., Velcheva, I., Stoyanova, S. and Georgieva, E., 2015. Fish in Ecotoxicological Studies. *Ecologia balkanica*, *7*(1).

Yoshino, M., Ito, M., Haneda, M., Tsubouchi, R., Murakami, K., 1999. Prooxidant action of aluminum Ion – Stimulation of iron-mediated lipid peroxidation by aluminum. Biometals;12:237–240. https://doi.org/10.1023/A:1009238732287.

Supplementary Material

Materials and Methods

Detailed Experimental Procedures for Data Presented in the Main Text.

2.4 Locomotor activity

ToxTrac is a computer vision framework for studies in animal behaviour. It uses an advanced image-based tracking that can handle several organisms. The module enables demarcation of regions of interest (ROIs). The software ToxTrac quantified motion in the video feed by reading the video stream frame-by-frame and calculating the differences in pixels between adjoining frames. The amount of movement in each ROI is recorded as the number of pixels that are different in the two subsequent frames, and the time at which this difference occurs is also recorded. Thus, both the magnitude and timing of each movement are quantified and saved in a matrix.

2.6 Sample collection

For biochemical analysis, samples of each experimental group were collected separately in 1.5 mL microfuge tubes, washed in cold phosphate buffer saline and homogenized in cold buffer (0.32 mM of sucrose, 20 mM of HEPES, 1 mM of $MgCl_2$, and 0.5 mM of phenylmethyl sulfonylfuoride (PMSF) that was prepared in ethanol to prevent protein degradation, pH 7.4) using an automated homogenizer (TissueLyser II, Qiagen, Hilden, Germany) The homogenate was centrifuged at 15000 x g at 4 °C for 20 min and the supernatant was transferred to new tubes and stored at -80 °C until analysis. For metallothioneins (MTs) analysis, the samples were collected separately and obtained in homogenization buffer (0.5 M sucrose, 20 mM Tris-HCl buffer, pH 8.6, containing 0.01 % β-mercaptoethanol) in 1.5 mL microfuge tubes. The samples were homogenized using a pellet mixer and cordless motor (VWR International, Carnaxide, Portugal). Aliquots were stored at -20 °C until analysis.

2.7 ROS accumulation and determination of ATPase activity

To 10 µL of sample homogenate, 100 µL of PBS (pH 7.4) and 8.3 µL DCFH-DA (dichlorofuorescein-diacetate 10 mg/mL in DMSO) were added and incubated for 30 min at 37 °C. After diffusion into

cells, DCFH-DA is deacetylated by cellular esterases and oxidized by ROS into the fluorescent compound dichlorofluorescein (DCF). At the end, the fluorescence was measured in a Varian Cary Eclipse spectrofluorometer (Varian, Palo Alto, USA) equipped with microplate reader at 485 nm and 530 nm excitation and emission wavelengths, respectively, against a reagent blank. All samples were performed in duplicate and ROS accumulation was estimated based on a DCF standard curve (0–500 µM). To determination of ATPase activity, 10 µL of samples was added to 20 µL of buffer (10 mM ouabain, 40 mM Tris, 80 mM NaCl, 8 mM $MgCl_2$ and 1 mM EDTA, pH 7.5) and pre-incubated for 10 min at 37 °C. The reaction was started by the addition of 10 µL ATP (4 mM) and incubated for another 30 min at 37 °C. The reaction was stopped by adding 200 µL of colour solution (1:6 of ascorbic acid to ammonium heptamolybdate, dissolved in H_2SO_4) and incubated for an additional 30 min at 37 °C. The inorganic phosphate present in the reaction media were measured at a wavelength of 820 nm and concentrations were determined by a calibration inorganic phosphate curve (0-10 mM Na_2HPO_4). Finally, the activities were expressed in µmol Pi/mg protein.

2.8 Enzyme activities

To evaluate the Superoxide dismutase (SOD), in each well of the 96-well microplate, 10 µL of sample were mixed with 170 µL of Potassium phosphate buffer (50 mM, pH7.4) supplemented with 0.6 mM hypoxanthine, 1 mM EDTA, and 0.2 mM NBT (nitrobluetetrazolium). The reaction was started by the addition of 20 µL of 0.5 U/mL xanthine-oxidase. Its activity was assayed by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT) at 560 nm. The increase in absorbance due to dismutation of O^{2-} into H_2O_2 was recorded for 3 min and SOD from bovine erythrocytes was used for construction of a standard curve (0–60 U/mL). For CAT activity, 10 µL of sample reacted with 90 µL of sodium buffer (100 mM, pH 7.4) containing 20 mM H_2O_2 and the decrease in absorbance was monitored at 240 nm for 3 min. Activity was calculated as enzyme units per milligram of protein using bovine catalase as a standard (0-6 U/mL). GPx activity was determinated by the reaction mixture (210 µL) contained 10 µL of sample, 100 mM phosphate buffer (pH 7.4), 5 mM NaN_3 (to inhibit CAT), 0.2 mM NADPH, 1 mM EDTA, 0.9 U/mL Glutathione

Reductase (GR) and 1.8 mM reduced glutathione. The reaction was started after the addition of fresh H_2O_2 (1.5 mM). The decrease in absorbance at 340 nm due to the oxidation of NADPH to $NADP^+$ was observed for 5 min. The activity was determined using the extinction coefficient (ε) of 6.22 m/M·cm. To evaluate the activity of GST, at 10 µL of sample it was added 180 µL of phosphate buffer 100 mM, pH 7.4 contaning 1 mM of CDNB (1-Chloro-2,4-nitrobenzene). The reaction was started by the addition of 25 mM of GSH and the absorbance was measured at 340 nm for 3 min. The activity was calculated using the extinction coefficient (ε) of 9.60 m/M·cm. To LDH determination, the assay system contained 10 µL of sample, 200 µL NADH (0.24 mM) and the reaction was started by the addition of 10 mM sodium pyruvate. The decrease in the absorbance due to the oxidation of NADH was monitored at 340 nm and the activity was calculated using the extinction coefficient (ε) of 6.22 m/M·cm. To evaluate the Ache, at 10 µL of enzymatic samples it was added the reaction buffer consisting of 180 µL of 0.5 mM DTNB (5,50-dithiobis-(2-nitrobenzoic acid)) in 0.05 M Tris buffer (pH 7.4). The reaction was started by the addition of 20 mM of acetylthiocholine iodide and the increase in the absorbance was measured at 405 nm for 3 min. The specific activity was determined using the TNB extinction coefficient (ε) of 13.6 m/M·cm.

2.9 Glutathione levels and oxidative stress index

To measure GSH, it was added 180 µL of 100 mM sodium phosphate buffer, pH 7.4, 5 mM EDTA and OPA to each 10 µL of sample. After 15 min of incubation at room temperature, the fluorescence was measured and GSH concentration was estimated based on a GSH standard curve (0–500 µM). For GSSG, 10 µL of sample was mixed with 90 µL of NaOH 0.1 N solution and 0.04 M of NEM (N-ethylmaleimide) and were incubated at room temperature for 30 min. Subsequently it was added 10 µL of OPA and incubated for other 15 min. In the end, the fluorescence of samples was measured and GSSG concentration was assessed based on a GSSG standard curve (0–500 µM).

2.10 Oxidative damage biomarkers and metallothioneins assay

To TBARS determination, 70 µL of distillated water and 50 µL of phosphate buffer 50 mM pH 7.4 were added to 10 µL of each sample. Subsequently, they were mixed with 1 mM BHT (butylated

hydroxytoluene) to prevent artificial lipid peroxidation and a solution of TBA reagent. TBA reagent was made from thiobarbituric acid (75 µL of TBA 1.3 % w/v) dissolved in 0.3 % NaOH and 50 µL of TCA 50 % (w/v). The mixture was incubated at 60 °C for 40 min and left to cool in ice at room temperature for 15 min after which 10 µL of SDS 20 % (sodium dodecyl sulfate) were added. The absorbance at 530 nm minus the absorbance at 600 nm was read using the PowerWave XS2 microplate scanning spectrophotometer (Bio-Tek Instruments, USA). The TABARS concentration was assessed based on a malondialdehyde (MDA) standard curve (0-500 µM). To CO determination, 15 µL of homogenate was incubated with an equal volume of 10 mM DNPH (in 2.5 N HCl) at room temperature for 10 min. Next, 7.5 µL of sodium hydroxide (6 N) was added to the mixture and incubated again for 10 min at room temperature. The absorbance of samples was measured at 450 nm using a PowerWave XS2 microplate scanning spectrophotometer (Bio-Tek Instruments, USA) and expressed as nmol of carbonyls/mg protein. The results were calculated assuming the absorption coefficient of (ε) 22.308 m/M·cm. To metallothioneins assay, the homogenates of samples were taken from -20 °C and centrifuged at 15000 xg for 20 min at 4 °C to obtain a supernatant containing metallothionein. To each 300 µL of supernatant, it was added 340 µL of a solution (1:13 absolute ethanol and chloroform) and centrifuged (4 °C) at 6000 xg for 10 min. Subsequently, 3 volumes of cold ethanol were added to the resulting supernatant and stored at -20 °C for 1 h. After another centrifugation step, the resulting pellets were washed with ethanol:chloroform:homogenization buffer (87:1:12) and then centrifuged again at 6000 xg for 10 min to metallothionein purification. The dried pellet was resuspended in Ellman's solution ((NaCl 2 M; 5,5'-d ithiobis (nitrobenzoicacid) 43 mM in phosphate buffer 0.2 M, pH 8)), adding 50 µL EDTA-HCl and 50 µL of NaCl 250 mM. In the end, the concentration of metallothioneins was evaluated by reading the absorbance at 412 nm in a PowerWave XS2 microplate scanning spectrophotometer (Bio-Tek Instruments, USA). A standard curve with GSH was created as a standard reference for a correct quantification of MTs in the samples.

5. Conclusion

This study proved that exposure to Al can affect a range of biological processes that impact on the health of the zebrafish fish both in adulthood and during its embryonic development. Al can influence both swimming activity and behaviour but also the physiology of the organism, compromising the histology and functionality of different organs such as the brain, muscles and gills.

The most severe effects were recorded at the lowest concentrations tested and at the shortest exposure time. Instead, high concentrations or long exposures induced improvement/stabilization of the conditions probably due to the activation of an adaptive mechanism already manifested by zebrafish after exposure to various pollutants.

Moreover, this study underlines the effectiveness of zebrafish as a model organism both as an adult and during its embryonic development, since it has perfectly adapted to the set objectives, allowing to obtain valuable information both in the ecotoxicological field and for translational toxicity studies.

Indeed, thanks to its use, it was shown that Al is both a dangerous pollutant, capable of altering the health of aquatic organisms, and a potential neurotoxic factor, capable of inducing neurodegeneration, alteration of myelin integrity and of GFAP expression.

Furthermore, the involvement of this metal in the deregulation of gene expression of marker linked to Parkinsonism, highlights that it can be involved in the development of neurological conditions potentially dangerous for human health.

The overall information gives a realistic snapshot of Al toxicity and provides new information on the mechanism of action of this metal, as widespread as dangerous for environmental and human health.

References

Agency for Toxic Substances and Disease (ATSDR), 2008. Toxicological Profile for Aluminum. CAS: 7429-90-5.

Agrawal, S., Flora, G., Bhatnagar, P., Flora, S.J., 2014. Comparative oxidative stress, metallothionein induction and organ toxicity following chronic exposure to arsenic, lead and mercury in rats. Cell Mol. Biol. (Noisy-le-grand) 60 (2), 13–21.

Arain, M.S., Afridi, H.I., Kazi, T.G., Talpur, F.N., Arain, M.B., Kazi, A., Arain, S.A., Ali, J., 2015. Correlation of aluminum and manganese concentration in scalp hair samples of patients having neurological disorders. Environ. Monit. Assess.; 187(2):10. DOI: 10.1007/s10661-014-4172-0.

Babin, P.J., Goizet, C., Raldúa, D., 2014. Zebrafish models of human motor neuron diseases: advantages and limitations. Prog Neurobiol.; 118:36-58. DOI: 10.1016/j.pneurobio.2014.03.001.
Best, J.D., Alderton, W.K., 2008. Zebrafish: an in vivo model for the study of neurological diseases. Neuropsychiatr. Dis. Treat. 4 (3), 567e576.

Bjorklund, G., Stejskal, V., Urbina, M.A., Dadar, M., Chirumbolo, S., Mutter, J., 2018. Metals and Parkinson's Disease: Mechanisms and Biochemical Processes. Curr Med Chem.; 25(19):2198-2214. DOI: 10.2174/0929867325666171129124616.

Bondy, S.C., 2016. Low levels of aluminum can lead to behavioral and morphological changes associated with Alzheimer's disease and age-related neurodegeneration. Neurotoxicology; 52:222-9. DOI: 10.1016/j.neuro.2015.12.002.

Cardwell, A.S., Adams, W.J., Gensemer, R.W., Nordheim, E., Santore, R.C., Ryan, A.C., Stubblefield, W.A., 2018. Chronic toxicity of aluminum, at a pH of 6, tofreshwater organisms: Empirical data for the development of interna-tional regulatory standards/criteria. Environ. Toxicol. Chem.; 37:36–48. DOI:10.1002/etc.3901.

Colomina, M.T., Peris-Sampedro, F., 2017. Aluminum and Alzheimer's Disease. Adv Neurobiol. 2017;18:183-197. DOI: 10.1007/978-3-319-60189-2_9.

Exley, C., 2006. Aluminium and iron, but neither copper nor zinc, are key to the precipitation of beta-sheets of Abeta42 in senile plaque cores in Alzheimer's disease. J. Alzheimers Dis.; 10(2-3):173-7. DOI: 10.3233/jad-2006-102-305.

Exley, C., House, E.R., 2011. Aluminium in the human brain. Monatsh Chem. 142(4):357-63. https://doi.org/10.1007/s00706-010-0417-y.

Favorito, R., Chiarelli, G., Grimaldi, M.C., De Bonis, S., Lancieri, M., Ferrandino, I., 2011. Bioaccumulation of cadmium and its cytotoxic effect on zebrafish brain. Chem. Ecol.; 27(2),39-46. https://doi.org/10.1080/02757540.2011.625937.

Gensemer, R.W., Gondek, J.C., Rodriquez, P.H., Arbildua, J.J., Stubblefield, W.A., Cardwell, A.S., Santore, R.C., Ryan, A.C., Adams, W.J., Nordheim, E., 2018. Evaluating the effects of pH, hardness, and dissolved organic carbon on the toxicity of aluminum to freshwater aquatic organisms under circumneutral conditions. Environ. Toxicol. Chem.; 37(1):49-60. DOI: 10.1002/etc.3920.

Guo, S., 2004. Linking genes to brain, behavior and neurological diseases: what can
we learn from zebrafish? Genes Brain Behav. 3 (2), 63e74. https://doi.org/10.1046/j.1601-183X.2003.00053.x

Jagoe, C.H., Haines, T.A., 1997. Changes in gill morphology of Atlantic salmon (Salmo salar) smolts due to addition of acid and aluminum to stream water. Environ. Pollut.; 97(1-2):137-46. DOI: 10.1016/s0269-7491(97)00069-9.

Jones, K., Linhart, C., Hawkins, C., Exley, C., 2017. Urinary Excretion of Aluminium and Silicon in Secondary Progressive Multiple Sclerosis. EBioMedicine.; 26:60-67. DOI: 10.1016/j.ebiom.2017.10.028.

Kawahara, M., Kato-Negishi, M., 2011. Link between Aluminum and the Pathogenesis of Alzheimer's Disease: The Integration of the Aluminum and Amyloid Cascade Hypotheses. Int. J. Alzheimers Dis.; 2011:276393. DOI: 10.4061/2011/276393.

Klein, G.L., 2019. Aluminum toxicity to bone: A multisystem effect? Osteoporos Sarcopenia;5(1):2-5. DOI: 10.1016/j.afos.2019.01.001.

Kongerud, J., Søyseth, V., 2014. Respiratory disorders in aluminum smelter workers. J. Occup. Environ. Med.; 56(5 Suppl):S60-70. DOI: 10.1097/JOM.0000000000000105.

Krewski, D., Yokel, R.A., Nieboer, E., Borchelt, D., Cohen, J., Harry, J., Kacew, S., Lindsay, J., Mahfouz, A.M., Rondeau, V., 2007. Human health risk assessment for aluminium, aluminium oxide, and aluminium hydroxide. J. Toxicol. Environ. Health B Crit. Rev.; 10 Suppl 1(Suppl 1):1-269. DOI: 10.1080/10937400701597766. Erratum in: J Toxicol Environ Health B Crit Rev.;11(2):147.

Lanzarin, G.A.B., Félix, L.M., Santos, D., Venâncio, C.A.S., Monteiro, S.M., 2019. Dose-dependent effects of a glyphosate commercial formulation-Roundup® UltraMax - on the early zebrafish embryogenesis. Chemosphere, 223,514–522. DOI:10.1016/j.chemosphere.2019.02.071.

Lawrence, G.B., Sutherland, J.W., Boylen, C.W., Nierzwicki-Bauer, S.W., Momen, B., Baldigo, B.P., Simonin, H.A., 2007. Acid rain effects on aluminum mobilization clarified by inclusion of strong organic acids. Environ. Sci. Technol. 41 (1), 93–98. https://doi.org/10.1021/es061437v.

Monaco, A., Capriello, T., Grimaldi, M.C., Schiano, V., Ferrandino I., 2017a. Neurodegeneration in zebrafish embryos and adults after cadmium exposure. Journal List. Eur. J. Histochem.; 61(4); 19PMC5656802. DOI: 10.4081/ejh.2017.2833.

Monaco, A., Grimaldi, M.C., Ferrandino, I., 2016. Neuroglial alterations in the zebrafish brain exposed to cadmium chloride. J. Appl. Toxicol.; 36(12),1629-38. DOI: 10.1002/jat.3328.

Monaco, A., Grimaldi, M.C., Ferrandino, I., 2017b. Aluminium chloride-induced toxicity in zebrafish larvae. J. Fish. Dis.; 40(5), 629-35. DOI: 10.1111/jfd.12544.

Morris, G., Puri, B.K. & Frye, R.E., 2017. The putative role of environmental aluminium in the development of chronic neuropathology in adults and children. How strong is the evidence and what could be the mechanisms involved?. Metab. Brain Dis. 32;1335:1355 . https://doi.org/10.1007/s11011-017-0077-2.

Orger, M.B., de Polavieja, G.G., 2017. Zebrafish behavior: opportunities and challenges. Annu. Rev. Neurosci. 40, 125–147. https://doi.org/10.1146/annurev-neuro-071714-033857.

Pereira, S., Cavalie, I., Camilleri, V., Gilbin, R., Adam-Guillermin, C., 2013. Comparative genotoxicity of aluminium and cadmium in embryonic zebrafish cells. Mutat Res. 20;750(1-2):19-26. DOI: 10.1016/j.mrgentox.2012.07.007.

Poléo, A.B.S., Schjolden, J., Sørensen, J., Nilsson, G.E., 2017. The high tolerance to aluminium in crucian carp (Carassius carassius) is associated with its ability to avoid hypoxia. PLoS One; 12(6):e0179519. DOI: 10.1371/journal.pone.0179519.

Spence, R., Gerlach, G., Lawrence, C., Smith, C., 2008. The behaviour and ecology of the zebrafish, Danio rerio. Biol Rev Camb Philos Soc.; 83(1):13-34. DOI: 10.1111/j.1469-185X.2007.00030.x.

Spitsbergen, J.M., Kent, M.L., 2003. The state of the art of the zebrafish model for toxicology and toxicologic pathology research--advantages and current limitations. Toxicol. Pathol. 2003 Jan-Feb;31 Suppl(Suppl):62-87. DOI: 10.1080/01926230390174959.

Tierney, K.B., 2011. Behavioural assessments of neurotoxic effects and neurodegeneration in zebrafish. Biochim Biophys Acta; 1812(3):381-9. DOI: 10.1016/j.bbadis.2010.10.011.

WHO, 213. Guidelines for Drinking-water Quality; Aluminium in drinking-water. WHO/HSE/WSH/10.01/13.

WHO, Geneva, Switzerland, 1997. Environmental health criteria for aluminium. Environmental Health Criteria No.194 pp.xx + 282 pp. ref.many. ISSN: 0250-863X.

Yang, L., Ho, N.Y., Alshut, R., Legradi, J., Weiss, C., Reischl, M., Mikut, R., Liebel, U., Müller, F., Strähle, U., 2009. Zebrafish embryos as models for embryotoxic and teratological effects of chemicals. Reprod Toxicol.; 28(2):245-53. DOI: 10.1016/j.reprotox.2009.04.013.

Yokel, R.A., 2020. Aluminum reproductive toxicity: a summary and interpretation of scientific reports. Crit. Rev. Toxicol.; 50(7):551-593. DOI: 10.1080/10408444.2020.1801575.

Zhang, J., Lyu, Z., Shao, S., Fang fang Li, F., Yang, S., Song, W., Li, W., Li, S., 2016. Effects of aluminum toxicity induced by acid deposition on pine forest ecosystem in Longli of Guizhou Province, Southwestern China. Chin. Geogr. Sci. 26, 495–507. https://doi.org/10.1007/s11769-015-0763-0.

Zon, L.I., 1999. Zebrafish: a new model for human disease. Genome Res.; 9(2):99-100.