



Dietary solutions for aluminum embryotoxicity: A study in *Danio rerio* using spirulina and okra-spirulina diets

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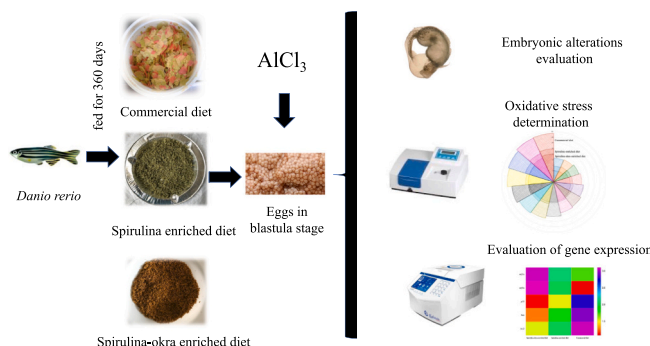
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HIGHLIGHTS

- Spirulina-okra diet boosts oocyte viability.
- Diets spirulina-okra & spirulina reduce aluminum-induced embryonic malformations.
- Antioxidant genes are upregulated by these diets.
- Spirulina-okra & spirulina diets mitigate aluminum-induced embryotoxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

Aluminum (Al) is a versatile element commonly employed in various industries and water treatment processes. However, its presence in aquatic ecosystems can elicit adverse effects on organisms, particularly the *Danio rerio* fish species. Aluminum exposure has been associated with a spectrum of issues, ranging from oxidative stress to behavioral anomalies, reproductive disruptions, and morphological alterations in these organisms. This research aimed to assess the impact of aluminum chloride (AlCl_3) on *D. rerio* embryos and explore strategies to mitigate its effects. Three dietary groups (commercial, okra-spirulina, and spirulina) were studied, focusing on embryonic development, oxidative damage, and gene expression changes. The study revealed that diets enriched with spirulina and okra-spirulina effectively reduced aluminum-induced embryotoxicity, oxidative stress, and gene expression alterations, surpassing the commercial diet. However, all AlCl_3 -exposed groups experienced adverse effects on embryonic development, including hatching anomalies, structural deformities, and cardiac delays. The okra-spirulina group showed milder toxic responses. In conclusion, this study highlights the potential of spirulina and okra-spirulina diets in mitigating aluminum-triggered oxidative stress and apoptosis in *D. rerio*. It

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underscores the need for future research on embryonic development and carries significant implications for environmental conservation and the well-being of aquatic organisms in aluminum-contaminated environments.

1. Introduction

Embryonic development is a process susceptible to a variety of environmental and chemical factors such as changes in diet or exposure chemicals or viruses, radiation, or hyperthermia, as documented in previous research (Hamilton et al., 2016; Pannetier et al., 2020). Among these factors, aluminum, a ubiquitous metal within many aquatic ecosystems, has emerged as a significant concern, impacting a wide spectrum of wildlife, including the extensively studied *D. rerio* (Battaglin et al., 2023; Naz et al., 2023). Zebrafish, well-established in scientific research, serve as model organisms, providing insights into the repercussions of toxic substances like aluminum on embryonic development (Chen et al., 2023; Nellore, 2023).

Exposure to aluminum at concentrations exceeding 1–5 mg/L has been linked to adverse health effects in both human and animal populations, including neurotoxicity, reproductive challenges, and notably, embryotoxicity (Abd Elnabi et al., 2023; Zhang et al., 2023). The latter, with its potential for long-term consequences on fish populations and the broader aquatic food chain, is of particular concern (Li et al., 2023). However, understanding the multifaceted mechanisms underlying aluminum's toxicity remains a complex task. This metal interacts with sulfhydryl groups in amino acids, such as cysteine and methionine, forming adducts with nitrogenous bases, subsequently leading to DNA destabilization (Bajaj et al., 2023; Fu, 2023). Additionally, aluminum indirectly stimulates the generation of reactive oxygen species (ROS), thereby inciting oxidative stress (Abdalla et al., 2019; Islam et al., 2023). Given the diverse facets of its impact, finding effective strategies to mitigate aluminum's embryotoxic effects is crucial, bearing profound implications for biodiversity conservation and food safety.

Within this context, substantial efforts have been dedicated to counteracting the detrimental effects of aluminum on embryonic development (L. Wang et al., 2023; Y.Y. Wang et al., 2023). One promising approach involves specialized diets enriched with natural ingredients known for their protective properties (Matera et al., 2023; Waliat et al., 2023). Of particular note among these ingredients are spirulina (*Arthrospira maxima*) and okra (*Abelmoschus esculentus*), which have garnered significant attention due to their potential mitigating properties (Abdel-Razek et al., 2023; Abidi et al., 2023; Nasirin et al., 2023; Pérez-Alvarez et al., 2023; Phoswa and Mokgalaboni, 2023). Spirulina, a microalga, is rich in protein, vitamins, and minerals, known for its well-documented antioxidant and anti-inflammatory properties (Zanella and Vianello, 2023). In contrast, okra, a plant celebrated for its fiber and antioxidant content, has demonstrated protective efficacy in various biological models (Guebebia et al., 2023).

This work adds a new dimension to pre-existing findings by delving into the molecular mechanisms underlying oxidative stress and apoptosis in response to aluminum exposure, an area largely unexplored in previous studies. While previous research has laid the groundwork for understanding the impact of aluminum on embryonic development and its potential amelioration by dietary interventions, this study differs by highlighting the cellular responses elicited by aluminum toxicity. This novel aspect significantly advances our understanding of the biochemical aspects through which aluminum affects embryonic development, thus contributing to the formulation of precise strategies for the preservation of ecosystems and aquatic species exposed to this environmental pollutant.

The primary objective of this study is to evaluate the effectiveness of two innovative diets in mitigating aluminum-induced embryotoxicity in *D. rerio*. These diets, one predominantly incorporating spirulina and the other combining spirulina with okra, are hypothesized to counterbalance the detrimental effects of aluminum during the crucial phase of

embryonic development, ultimately mitigating oxidative stress and apoptosis in *D. rerio*. To test this hypothesis, a comprehensive series of experiments was conducted, involving the exposure of *D. rerio* embryos to sublethal aluminum concentrations, followed by feeding with the aforementioned diets.

In this paper, the results of the designed experiments will be presented, with the aim of providing a detailed understanding of how these diets may influence aluminum-induced embryotoxicity, oxidative damage and apoptosis in *D. rerio*. The implications of these findings will enrich the understanding of how dietary interventions can modulate the responses of aquatic organisms to aluminum exposure, contributing to effective environmental protection and species conservation strategies.

2. Materials and methods

2.1. Ethical approval

The research protocol obtained approval from the Ethics and Research Committee at the Autonomous University of the State of Mexico (UAEM). Approval, identified by RP. UAEM. ERC.156.2022, validates that all experiments conducted in this study conform to the institution's stringent animal care standards. Furthermore, adherence to the Mexican official standard governing the breeding, care, and utilization of laboratory animals, NOM-062-ZOO-1999, was conscientiously observed and followed.

2.2. Chemical reagents

We procured the aluminum chloride standard (99 %, AlCl_3) and all other necessary reagents from Sigma-Aldrich, based in St. Louis, MO. Our supply of spirulina algae (*Arthrospira maxima*) was obtained from Spiral Spring, located in Puebla, Mexico, while the okra (*Abelmoschus esculentus*) used in our study was sourced from PeachDish (Atlanta, Georgia). The experimental setup utilized nominal aluminum concentrations derived from a standard aluminum chloride solution. These carefully calibrated solutions spanned a range of concentrations, including 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, and 8000 $\mu\text{g/L}$. The precise control of these concentrations ensured the comprehensive evaluation of aluminum's impact on embryonic development in *D. rerio*, fostering an accurate understanding of its effects across a spectrum of environmentally relevant levels.

2.3. Diet preparation

In the process of elaboration of the experimental diets, all the ingredients described in Table 1 were meticulously combined. To achieve a complete and uniform mixture, a grain mill was used, ensuring continuous homogenization for approximately 5 min or until complete incorporation of the ingredients was achieved. Following the initial mixing step, 5 mL of 1 % sodium alginate solution, a food additive recognized for its thickening, foaming, and gelling properties, was introduced to the mixture containing all powdered components. This addition aimed to achieve a homogeneous texture throughout the mixture. Subsequently, the mixture underwent a sieving process using a mesh with an #80 aperture size to attain a consistent and fine-textured consistency. Once the sieving was complete, the prepared mixture was evenly spread into trays. These trays were then carefully placed inside ovens, where they were subjected to a controlled temperature of 55 °C for a duration of 12 h. This step was crucial to facilitate the desired solidification and texture of the diets. The proximal composition of the

basal diet was determined through the aforementioned sieving process. For the spirulina-enriched diet, the spirulina-okra mixture diet, and the commercial diet, their respective proximate compositions were assessed using the established methods endorsed by the Association of Official Analytical Chemists International (AOAC). It's important to highlight that our formulated diet adheres to the recommended nutritional requirements for fish, as stipulated by the National Research Council (NRC). This ensures that the dietary regimens provided to our zebrafish subjects meet the necessary nutritional standards for their optimal health and development within the scope of our study.

Table 1 shows the detailed composition and proximate chemical composition of the different diets used in this study. This information provides critical insights into the nutritional content and makeup of the foundational dietary regimen utilized in the experiments. This serves as a benchmark for understanding the dietary factors on which this research is based, ensuring that the diets fed to the zebrafish were consistent and well defined throughout the study.

2.4. Care of zebrafish

Under controlled laboratory conditions, a population of four hundred and fifty adult zebrafish of the AB strain was housed. The fish were carefully distributed into three separate 100-liter tanks, containing water previously UV-sterilized and charcoal-filtered, at a constant temperature of 28 ± 1 °C. The fish were maintained on a constant light/dark cycle, with 14 h of light and 10 h of dark per day. Over a 360-day exposure duration, the fish were consistently subjected to the three dietary regimens as described earlier. Out of the 450 fish in the study, they were divided into three equal groups for feeding: one-third received the spirulina-enriched diet, another third were fed the diet comprising a combination of spirulina and okra, and the final third were provided with the commercial diet (Lomas Wardley).

In order to uphold optimal water quality within the three tanks, the water medium was refreshed every two days. All parameters were monitored and controlled during zebrafish maintenance and breeding, as well as during subsequent experiments. Water quality parameters are shown in Table 2.

Table 1
Composition and proximate chemical composition of the diets.

	Functional diet (g)	Formulation of the spirulina-added diet (%)	Functional diet (g)	Formulation of the Spirulina-okra-added diet (%)
Fish meal (<i>Engraulis encrasicolus</i>)	50	67.1	50	64.5
Fish muscle (<i>Oreochromis</i> sp.)	10	13.4	10	12.9
Fish oil	1.4	1.9	1.4	1.8
Soybean meal	10	13.4	10	12.9
<i>Arthrospira maxima</i>	1	1.3	1	1.3
<i>Abelmoschus esculentus</i>	0	0	3	3.9
Vitamin and mineral premix ^a	2.1	2.9	2.1	2.7
Total dry matter contains	74.5	100	77.5	100

Calculated chemical analysis	Commercial diet	Spirulina-based diet	Spirulina-okra -based diet
Dry matter (%)	100	100	100
Crude protein	43	62.8	55.8
Crude fiber	15.9	2.0	3.4
Carbohydrates	36	9.9	16.9
Ash	12.0	13.2	12.8
Lipid	5.0	8.4	6.5
Moisture	9.0	3.8	4.7
Grass energy (kcal 100 g ⁻¹ diet)	361	366	349

^a Composition of vitamins and minerals premix g 100 g⁻¹: Ascorbic acid 23.7 g; biotin 7.5 mg; boron citrate 63.6 mg; calcium 5.7 g; choline (bitartrate of choline) 6.3 g; chromium (III) nicotinate 6.8 mg; copper gluconate 45.5 mg; magnesium 6.1 g; manganese gluconate 45.4 mg; molybdenum citrate 1.1 mg; potassium 0.6 g; potassium iodide 9 mg; selenium 4.5 mg; silicon 90.9 mg; sodium 0.3 g; tocopherol mixture 2 g; vanadium citrate 0.9 mg; vitamin A 26.2 mg; vitamin B1 0.7 g; vitamin B2 0.7 g; vitamin B3 1 g; vitamin B5 2 g; vitamin B6 0.8 g; vitamin B9 15.1 mg; vitamin B12 1.2 mg; vitamin D3 1 mg; vitamin E (D-alpha tocopherol succinate) 4.1 g; vitamin K1 1.5 mg; vitamin K2 1.5 mg; zinc 20.6 mg. Flavonoids mixture: *Camelia sinensis* extract 1.7 g; hesperidin 1 g; quercetin hydrochloride 5 g; resveratrol (*Polygonum cuspidatum*) 1 g; rutin 1 g. Carotenoids mixture: Alpha-carotene, gamma-carotene 10.1 mg; curcumin 1.4 g; lutein 15.1 mg; lycopene 25.2 mg. Antioxidants: Alpha-lipoic acid 2.5 g; coenzyme Q10 0.3 g; inositol 3.2 g; N-acetyl L-cysteine 3.6 g.

Table 2
Water quality parameters.

Parameter	Value ± SD
pH	7.35 ± 0.15
Hardness	95 ± 5 mg/L CaCO ₃
Un-ionized ammonia	0.009 ± 0.003 mg/L
Alkalinity	95 ± 5 mg/L CaCO ₃
Nitrate (NO ₃ ⁻)	3.1 ± 0.2 mg/L
Nitrite (NO ₂ ⁻)	0.030 ± 0.010 mg/L
Dissolved oxygen	10.5 ± 0.5 mg/L
Conductivity	390 ± 35 µS/cm
Salinity	0.7 ± 0.2 g/L

Data represents the medium values of all replicates from all experiments ± SD.

2.5. Zebrafish embryo collection

On the evening prior to spawning, adult male and female zebrafish, each measuring 4–5 cm in size and adhering to a 2:1 male-to-female ratio, were carefully chosen from the tanks in which they were raised under the three distinct dietary regimens. Subsequently, they were individually placed in separate breeding chambers. The spawning was induced in the morning. Embryos were collected, at 1 hour post-fertilization (hpf), rinsed in embryo water and bleached according to established protocol (Elizalde-Velázquez et al., 2021a). Fertilized embryos were classified under a stereoscopic microscope according to standard methods (Kimmel et al., 1995) and middle blastula stage embryos (equivalent to 2.5 hpf) were selected. After the selection, middle blastula stage embryos were kept in an incubator (27 ± 1 °C) and left in water fortified with 60 µg of Instant Ocean® salts [Cl (18,740 mg/L), Na (10,454 mg/L), SO₄ (2631 mg/L), Mg (1256 mg/L), Ca (400 mg/L), K (401 mg/L), HCO₃ (154 mg/L), Sr (7.5 mg/L) and B (6 mg/L)] (Christy and Dickman, 2002) per milliliter until embryos reached sphere stage (4 hpf).

2.6. Fish embryo toxicity (FET) assessment

Seventy-two embryos at the sphere stage (4 hpf) were meticulously

selected and subsequently, they were randomly allocated into individual wells within 24-well plates, with one embryo placed per well. These plates were divided into two groups: one received a control solution consisting of ultrapure water, while the other was exposed to test solutions containing aluminum at environmentally relevant concentrations (1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500 and 8000 µg/L) (Weisner et al., 2023; He et al., 2021; Edzwald, 2020; Zhang et al., 2016; Górski and Siepak, 2014; Wang et al., 2011). To ensure the robustness and replicability of the results, each test solution of aluminum was distributed across three separate 24-well plates. Furthermore, the exposure of embryos to each distinct aluminum test solution was performed in triplicate, constituting three independent experiments. This experimental design was implemented to provide a solid foundation for the subsequent analyses and conclusions drawn from the study. The exposure was carried out in accordance with the established protocols outlined in [Test No. 236: Fish Embryo Acute Toxicity \(FET\) Test \(2013\)](#). The selection of aluminum concentrations was based on their environmental significance, considering data obtained from various sources such as influents and effluents of wastewater treatment plants, raw wastewater, surface water, drinking water, and hospital effluents (Weisner et al., 2023; He et al., 2021; Edzwald, 2020; Zhang et al., 2016; Górski and Siepak, 2014; Wang et al., 2011). This approach was taken to ensure that our experimental conditions closely mirrored real-world scenarios, enhancing the relevance and applicability of our findings to natural environmental conditions. The Fish Embryo Acute Toxicity (FET) test was performed using oocytes sourced from zebrafish that had been nourished with the three distinct dietary regimens under study. The determination of the sample size adhered to the guidelines specified in OECD 236, which recommend the use of 24-well plates for conducting this test. This rigorous adherence to established protocols ensures the validity and consistency of the experimental setup, facilitating the robust assessment of the dietary impact on the test subjects.

To minimize potential biases, several measures were carefully implemented: Firstly, the primary researcher responsible for the study was unaware of the specific treatments to which the embryos were exposed. Secondly, a dedicated laboratory technician managed the preparation of the test solutions and system maintenance. Thirdly, another lab technician was entrusted with the randomization process. This strategy maintained a double-blind approach in which lab technicians were unaware of treatment conditions during system upkeep, and researchers remained uninformed during the randomization process. The randomization procedure was executed as follows: The total number of embryos was divided into 15 groups, each containing 72 embryos, representing both the control solution and the various aluminum test solutions. Subsequently, each group was assigned a numerical identifier from 1 to 15 using the standard “=INT(RAND())” function in Microsoft Excel, with each number representing a distinct treatment condition to which the embryos were exposed. This approach ensured impartiality and robustness in the experimental design, reinforcing the credibility of the study's outcomes.

After a four-day exposure period (equivalent to 96 hpf), an evaluation of embryo mortality and malformation rates was performed. From the data collected, toxicity indicators were calculated, such as the teratogenic index of aluminum, the LC₅₀ (the lethal concentration affecting 50 % of embryos) and the EC_{50m} (the concentration inducing malformations in 50 % of embryos). IBM SPSS Statistics 22 software was employed for data analysis and to construct concentration-response curves, with a specific focus on aluminum-induced malformations in *D. rerio*. The malformation rate was determined as the percentage of embryos exhibiting at least one malformation, using the control group as the reference point for comparison. This method provided a quantitative evaluation of aluminum's impact on zebrafish embryo development.

2.7. Oxidative stress assessment

One gram of zebrafish oocytes in the sphere stage, were exposed to each of fifteen different concentrations of aluminum. This methodology was in accordance with the procedures previously established by [Elizalde-Velázquez et al., 2021b](#). These exposures were conducted in 4-liter tanks as per our experimental setup. Zebrafish embryos were sampled at two different times, namely at 72 hours post-fertilization (hpf) for one group and at 96 hpf for the other. These endpoint times were selected because at that time the zebrafish embryos had already hatched and their enzymatic system was already working ([Timme-Laragy et al., 2018](#)). All along the exposure period, temperature (27 ± 1 °C) and light/dark cycles (14:10) were kept constant in all the systems. For sampling, a mean of 0.5 g of embryos were randomly selected at 72 hpf and 96 hpf, and homogenized in 1 mL of phosphate buffer solution (pH 7.4). In order to minimize the biases in this research, we considered the following points: 1) the main researcher of this research would know the treatments at which embryos were exposed; 2) one lab technician would be in charge of preparing the test solutions and the maintenance of the systems; and 3) another lab technician would be in charge of the randomization process. This way, lab technicians were blinded to treatment during systems maintenance, while researchers were blinded to treatment during randomization. Randomization was performed as follows. Embryos from each system were divided into 30 groups (a mean of 100 embryos per group), and groups were assigned with a number between 1 and 2 using the standard =INT(RAND()) function in Microsoft Excel. This way, 1 represents the groups that were used at 72 hpf, while 2 symbolizes the groups used at 96 hpf. Lab technicians were blinded to treatment during systems maintenance, while researchers were blinded to treatment during randomization. Upon sample collection, the homogenized zebrafish embryos were divided into two separate Eppendorf tubes and subjected to centrifugation at 4 °C. In the first tube, which contained 300 µL of trichloroacetic acid (TCA) and 300 µL of the homogenate, centrifugation was carried out at 13,307 ×g. Meanwhile, the second tube, which held the remaining 700 µL of homogenate, underwent centrifugation at 15,722 ×g. We utilized the homogenate from the second tube to evaluate the activity levels of superoxide dismutase (SOD) and catalase (CAT). These assessments followed established protocols as detailed in the studies by [Misra and Fridovich \(1972\)](#), [Radi et al. \(1991\)](#), and [Gunzler and Flohe-Clairborne \(1985\)](#). For the first tube containing the sample, we assessed lipoperoxidation level (LPX), protein carbonyl content (POX), and hydroperoxides content (HPX) using guidance provided by [Buege and Aust \(1978\)](#), [Levine \(1994\)](#), and [Jiang et al. \(1992\)](#), respectively. To ensure accuracy and consistency, we determined the total protein content in each sample using the [Bradford \(1976\)](#) method. This protein content served as a reference for expressing the results of our oxidative stress biomarkers, enabling us to assess the impact of aluminum exposure on oxidative stress in zebrafish embryos. The oxidative stress experiment was conducted in triplicate, and we repeated this experiment three times, resulting in a total of nine sets of results for each biomarker. These multiple data points (n = 9) were subsequently utilized for statistical analyses and to generate charts.

2.8. Gene expression

2.8.1. RNA extraction

The procedure for zebrafish embryo exposure was as previously described in [Section 2.7](#). For RNA isolation, a total of 40 zebrafish embryos were collected at 96 hpf, immediately placed in RNA lysis solution, and rapidly frozen at -80 °C until total RNA isolation. RNA isolation was carried out using the RNeasy mini kit from Qiagen following the manufacturer's protocol. In brief, oocytes were homogenized in 600 µL of RLT lysis buffer, followed by a series of centrifugation steps and reagent additions. RNA was eluted from the spin column in 30 µL of nuclease-free water.

The isolated total RNA was quantified spectrophotometrically at 260

nm using the THERMO Scientific NanoDrop 2000/2000c kit. RNA purity was assessed by calculating the UV absorbance ratios at 260 nm, 230 nm, and 280 nm. This ensured the quality and integrity of the extracted RNA for subsequent analyses.

2.8.2. cDNA synthesis from total RNA

Before proceeding with the reverse transcription process, 1 µg of total RNA underwent a crucial step to eliminate genomic DNA (gDNA) contamination. This was achieved through the application of the gDNA Wipeout Buffer, an essential component of the procedure. By meticulously removing any residual gDNA, we ensured the specificity of our subsequent gene expression analysis.

Following this step, reverse transcription was initiated using the Quantitect® Reverse Transcription Kit from QIAGEN, a trusted provider of molecular biology reagents. This kit included the Quantiscript Reverse Transcriptase and Quantiscript Reverse Buffer, both instrumental in the accurate synthesis of complementary DNA (cDNA). The process adhered to the manufacturer’s instructions, maintaining precision and consistency. In brief, the reaction commenced with an incubation period of 15 min at 42 °C, promoting the conversion of RNA into cDNA. This was followed by a denaturation step, in which the reaction was held at 95 °C for 3 min, finalizing the synthesis of stable and amplifiable cDNA molecules. This cDNA was subsequently employed for gene expression analysis, allowing us to delve into the molecular responses of zebrafish embryos in the context of dietary interventions and aluminum exposure.

2.8.3. Real-time PCR for gene expression analysis

Following the synthesis of cDNA, a crucial step in the study involved gene expression analysis, employing Real-Time PCR. The cDNA products were diluted at a ratio of 1:3 in nuclease-free water, ensuring the appropriate concentration for downstream analysis. Subsequently, 2 µL of this diluted cDNA were introduced into each PCR reaction.

Relative quantification of *nrf1*, *nrf2*, *p53*, *bax* and *bcl2* genes was performed by real-time PCR using the Rotor-Gene Q system from Qia-gen. QuantiNova Sybr® Green was used, following the instructions provided by the manufacturer. The primers, critical components of the PCR process, were meticulously designed with precision using the Primer3 primer design software, adhering to the criteria defined by Bustin (2000) for real-time PCR primer design. Detailed information about primer sequences and GenBank accession numbers for all target genes can be found in Table 3. The primer concentration in each reaction was maintained at 500 nM.

The real-time PCR cycling conditions encompassed an initial denaturation step, conducted at 94 °C for 15 s, followed by 35 cycles, each cycle comprising denaturation at 94 °C for 15 s, hybridization at 60 °C for 30 s, and extension at 72 °C for 30 s. To ensure the consistency of our results and enable accurate comparisons, the internal β-actin gene served as the housekeeping gene and was systematically included in all samples.

At the culmination of the q-PCR cycles, a critical step involved the generation of a dissociation curve. This curve played a pivotal role in distinguishing specific PCR products from nonspecific ones, with precise conditions of 10 s at 95 °C, 10 s at 65 °C, and 10 s at 95 °C, featuring

0.2 °C increments during the melting curve phase, enabling data collection at each increment. After q-PCR reaction and melting curve analysis, the specificity of the PCR amplicons was validated. This critical step ensured the accuracy and precision of the gene expression analysis.

For each gene evaluated, the relative transcript level was determined and normalized using the reference gene β-actin. The transcript levels for each gene at different exposure concentrations were further normalized to their respective levels at each concentration. Relative fold change was calculated for each concentration using the equation: fold change = $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Here, $\Delta Ct = Ct_{(target\ gene)} - Ct_{(\beta-actin\ gene)}$; $\Delta\Delta Ct_{(concentrations)}$ symbolizes the difference in ΔCt values across different exposure concentrations compared to $\Delta\Delta Ct_{(concentration\ 1)}$.

2.9. Statistical analysis and significance testing

To evaluate and establish differences in means, we employed Dunnett’s multiple comparison test. Furthermore, a two-way ANOVA test was applied to ascertain the significance of variations among different concentrations. We conducted assessments of normality and homoscedasticity, utilizing the Shapiro-Wilk test to verify normal distribution and the Bartlett test to ensure uniform variance, respectively. It’s important to note that all oxidative stress biomarkers successfully met the criteria for normality. For the assessment of embryotoxic and teratogenic effects, we employed Fisher’s exact test. We considered results to be statistically significant when the p-value was <0.001, with all statistical analyses conducted using Sigma Plot 12.3 software. This comprehensive statistical approach allowed us to rigorously evaluate and establish the significance of our findings, ensuring robust and reliable outcomes in our study.

3. Results

3.1. Oocyte viability with different diets

Throughout the study, a comprehensive assessment was conducted to evaluate the viability percentages within each dietary group. As shown in Fig. 1, significant variations in zebrafish oocyte viability were observed among these dietary regimens. Notably, the diet enriched with a combination of spirulina and okra demonstrated the highest viability percentage, reaching an 95 %. Following closely, the diet solely enriched with spirulina displayed a viability rate of 89 %. In contrast, the group fed the commercial diet exhibited a comparatively lower viability percentage, recorded at 82 %.

3.2. Mortality and malformation rate

Table 4 presents a comprehensive summary of the mortality and malformation rates observed in *D. rerio* when subjected to varying concentrations of AlCl₃ across three distinct dietary groups. Our analysis revealed LC₅₀ values of 5205.2 µg/L for organisms on the commercial diet, 6121.8 µg/L for those on the spirulina-enriched diet, and 6873.9 µg/L for the spirulina and okra-enriched diet group. Furthermore, EC_{50m} values were calculated for each diet, resulting in values of 2150.6 µg/L

Table 3
Gene-specific primers sequences used in the present study.

Gen	Forward primer (5'-3')	Reverse primer (5'-3')	Access number
<i>nrf1</i>	CAGTGACAGTAGCCAGGTG	CTGACGCTTGTTGG	NM_001328540.1
<i>nrf2</i>	GGCGATCCTCTGTAAACCC	CCGAAGGATCCGTTTCGGT	NM_182889.1
<i>bax</i>	GGCTATTCAACCAAGGGTTCC	TGCGAATCACCAATGCTGT	AF231015
<i>p53</i>	GCGGATTGCTTTGTGGATG	CCGACCTCCTCTCACTAAA	NM_001328587
<i>bcl2</i>	TACGGGATGCTGGAGATGAA	CCAGITTCACCTCGTCTCTA	NM_001030253
<i>actβ1</i>	ACCACGCCGAAAGAGAAAT	ATGTCCACGTCGCACTTCAT	NM_131031

nrf1: nuclear factor erythroid 2-related factor 1; *nrf2*: nuclear factor erythroid 2-related factor 2; *bax*: BCL2 associated X; *p53*: tumor protein p53; *bcl2*: BCL2 apoptosis regulator; *actβ1*: actine β1.



Fig. 1. Dietary effects on oocyte viability in *D. rerio*.

Table 4
Mortality and malformation in *D. rerio* resulting from exposure to AlCl₃ after undergoing three distinct diets.

AlCl ₃ concentration (µg/ L)	Embryos exposed	Mortality				Malformations		
		Commercial diet	Spirulina- enriched diet	Spirulina okra- enriched diet		Commercial diet	Spirulina -enriched diet	Spirulina okra- enriched diet
Control	72	0 ± 0.1	0 ± 0.3	0 ± 0.1		0 ± 0.1	0 ± 0.4	0 ± 0.1
1500	72	1 ± 0.5	1 ± 0.8	1 ± 0.2		29 ± 0.9	3 ± 0.7	1 ± 0.5
2000	72	3 ± 0.7	3 ± 0.5	2 ± 0.6		37 ± 1.2	5 ± 0.6	3 ± 0.3
2500	72	7 ± 0.4	7 ± 0.6	4 ± 0.7		40 ± 1.6	8 ± 1.1	5 ± 1.2
3000	72	11 ± 0.9	10 ± 1.5	7 ± 1.1		44 ± 1.5	15 ± 1.7	8 ± 1.6
3500	72	14 ± 1.2	13 ± 0.8	10 ± 0.9		48 ± 2.2	23 ± 2.7	11 ± 2.2
4000	72	20 ± 1.6	17 ± 1.1	16 ± 1.4		53 ± 1.8	29 ± 1.9	13 ± 2.4
4500	72	26 ± 1.1	19 ± 1.3	18 ± 1.1		55 ± 2.3	34 ± 2.2	16 ± 1.7
5000	72	36 ± 1.6	26 ± 1.9	23 ± 1.7		57 ± 2.8	38 ± 2.9	19 ± 2.8
5500	72	39 ± 1.3	34 ± 1.7	25 ± 1.2		61 ± 2.5	45 ± 2.4	22 ± 2.5
6000	72	41 ± 1.7	37 ± 2.1	31 ± 1.9		66 ± 3.3	47 ± 2.5	27 ± 3.1
6500	72	45 ± 2.1	38 ± 2.6	34 ± 2.4		67 ± 2.9	49 ± 3.6	30 ± 3.8
7000	72	51 ± 2.5	41 ± 1.9	37 ± 2.9		69 ± 3.7	53 ± 3.9	36 ± 3.0
7500	72	55 ± 1.8	45 ± 2.8	40 ± 3.6		70 ± 3.1	55 ± 3.1	40 ± 3.4
8000	72	63 ± 2.2	49 ± 2.2	42 ± 1.9		70 ± 4.3	59 ± 3.8	43 ± 4.1
	LC ₅₀ =	5205.2 µg/L	6121.8 µg/L	6873.9 µg/L	EC _{50m} =	2150.6 µg/L	4749.7 µg/L	7257.5 µg/L
	CI=	4969.6–5460.5	5768.5–6553.2	6422.9–7465.6	CI=	1892.4–2380.9	4509.2–5005.2	6723.9–7987.2

TI (commercial diet) = 2.4.
TI (Spirulina-enriched diet) = 1.3.
TI (Spirulina okra-enriched diet) = 0.9.

for the commercial diet, 4749.7 µg/L for the spirulina-enriched diet, and 7257.5 µg/L for the spirulina and okra-enriched diet. Toxicological information was calculated, including the LC₅₀/EC_{50m} ratio, which revealed a teratogenicity index (TI) of 2.4 for the commercial diet, suggesting a higher potential for AlCl₃-induced teratogenicity compared to other diets. In contrast, the spirulina-enriched diet exhibited a TI of 1.3, while the spirulina and okra-enriched diet demonstrated a TI of 0.947, emphasizing variations in their susceptibility to AlCl₃-induced toxicity. These results signify the novel findings within the context of our study.

3.3. Main malformations identified with the different diets tested

Fig. 2 visually summarizes the major developmental alterations observed in each concentration group for the respective diets. These alterations encompass a spectrum of developmental irregularities, including pericardial edema, craniofacial malformations, growth retardation, kyphosis, lordosis, body hypopigmentation, yolk sac malformations, tail malformations, body malformations, hatching delays, among

others. In contrast, the control group displayed typical, unaffected development. Comparative analysis reveals a significantly higher incidence of malformations in *D. rerio* embryos from the commercial diet group when compared to those in the spirulina and spirulina-okra enriched diet groups. Particularly noteworthy is the spirulina-okra enriched diet, which demonstrated a statistically significant reduction in the proportion of malformations induced by AlCl₃ exposure (p < 0.05).

3.4. Evaluation of embryonic development

Fig. 3 presents a summary of the morphological developmental scores in *D. rerio* when exposed to the different diets investigated. Of note are the developmental alterations observed in the commercial diet group when subjected to varying concentrations of AlCl₃. On the contrary, embryos fed with diets enriched with spirulina or with the mixture of spirulina and okra showed fewer and less drastic alterations in their embryonic development in their embryonic development.

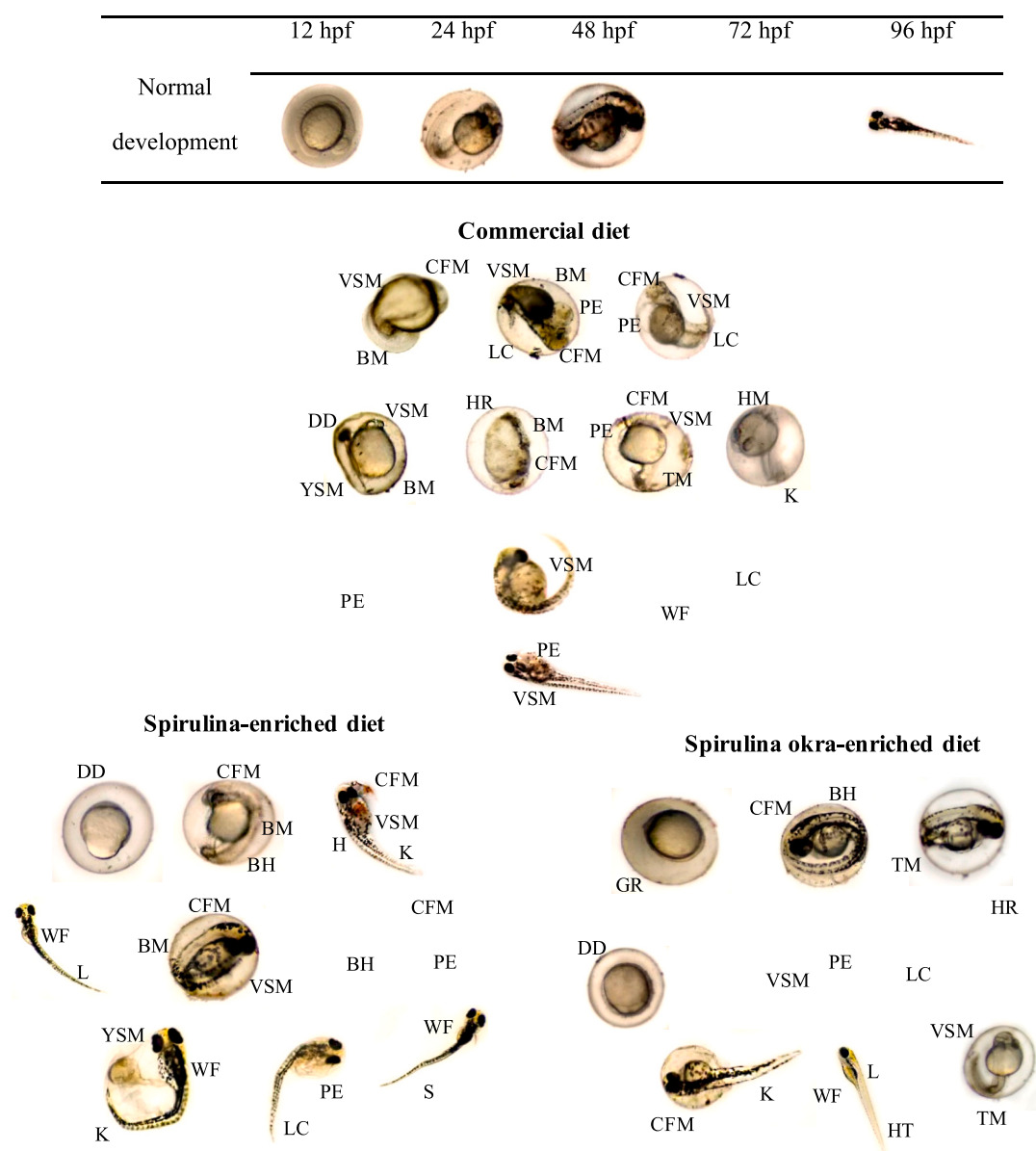


Fig. 2. Principal malformations observed in *D. rerio* following exposure to varying concentrations of AlCl_3 subsequent to undergoing three distinct diets.

PE = pericardial edema, CFM = craniofacial malformation, GR = growth retardation, K = kyphosis, L = lordosis, BH = body hypopigmentation, YSM = yolk sac malformation, TM = tail malformation, BM = body malformation, HR = hatching retardation, WF = without find, DD = developmental delay, VSM = vitelline sac malformation, S = scoliosis, H = hemorrhage, HT = hypopigmentation tail, LC = lordosis column, HM = head malformation.

3.5. Oxidative stress evaluation

3.5.1. Cellular oxidation biomarkers

Fig. 4 presents the levels of LPX, HPX, and POX at both the 72 hpf and 96 hpf. Clearly, all tested concentrations of AlCl_3 led to a significant increase in comparison to the control group. In contrast, diets enriched with either spirulina or the spirulina-okra showed a marked decrease in these oxidative stress markers when compared to the commercial diet. Remarkably, the diet enriched with spirulina and okra exhibited the most substantial reduction in these markers.

In Fig. 5, the activity of the antioxidant enzymes SOD and CAT at 72 and 96 hpf is depicted. Clear trends, showing a significant increase in the levels of these enzymes in embryos from organisms fed the commercial diet. To a somewhat lesser extent, the activity of these enzymes also increased in embryos from organisms fed diets enriched with spirulina and the spirulina-okra mixture.

3.6. Gene expression damage

Fig. 6 illustrates the impact of dietary composition on the expression of these genes. The outcomes derived from the commercial diet demonstrate that exposure to AlCl_3 for 96 hpf leads to a concentration-dependent upregulation in the expression of *nrf1*, *nrf2*, *p53*, *bax*, and *bcl2* genes. Notably, this figure also highlights the substantial protective effect exerted by both enriched diets (spirulina and spirulina-okra), extending its influence to transcriptional levels, resulting in a discernible decrease in the expression of all genes. Nevertheless, the presence of okra within the fish diet significantly diminishes the relative mRNA levels of these genes in comparison to other dietary regimens, even under the highest exposure concentration. This decline in gene expression can likely be attributed to the antioxidant properties of okra, which, in turn, reduces the transcriptional demands of the evaluated genes.

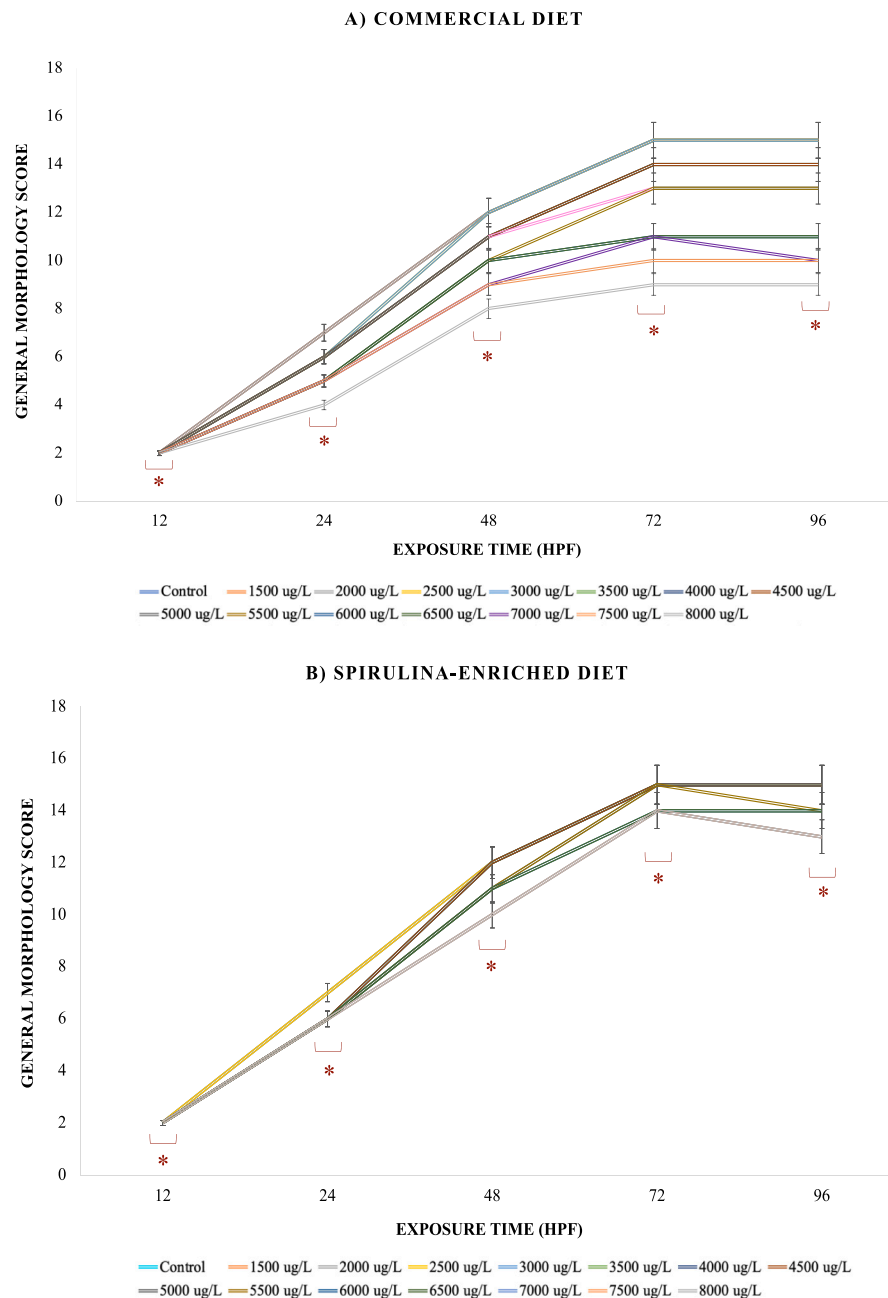


Fig. 3. General morphology scores of *D. rerio* embryos exposed to AlCl_3 at environmentally relevant concentrations. (*) Significant differences concerning the commercial diet group ($p < 0.001$). Data represents mean $\pm$ standard deviation (SD) ($n = 9$). From 24 hpf onwards, there are significant differences between the three treatments. 24 hpf: $F(44;360) = 5.78182$, $p < 0.001$; 48 hpf: $F(44;360) = 11.93939$, $p < 0.001$; 72 hpf: $F(44;360) = 26.33939$, $p < 0.001$; 96 hpf: $F(44;360) = 29.57576$, $p < 0.001$.

4. Discussion

The significantly higher viability of zebrafish oocytes observed in the spirulina-okra-enriched diet group (95 %) compared to both the spirulina diet group (89 %) and the commercial diet group (82 %) can be attributed to the unique nutritional composition of the okra-spirulina blend. Okra has been recognized for its compounds, which have been reported to possess coagulant properties against aluminum, along with phytochemicals that offer potential health benefits, as documented in recent studies (Bakhsh et al., 2023; Mohammadzadeh et al., 2023). When these nutritional components are combined with spirulina, a microalga well-known for its remarkable nutritional profile and

beneficial effects on aquatic organisms (Abreu et al., 2023; Al Mamun et al., 2023; Dianratri et al., 2023), they collectively formulate a diet that provides essential nutrients and bioactive compounds crucial for the reproductive health and oocyte viability of *D. rerio*.

Spirulina, abundant in protein, vitamins, minerals, and antioxidants, plays a pivotal role in supporting various aspects of fish reproductive processes (El-Sheekh et al., 2023; Pérez-Alvarez et al., 2023; Youssef et al., 2023). In contrast, the commercial diet may lack specific nutrients or bioactive components present in the spirulina-okra-enriched diet, potentially resulting in reduced oocyte viability (Abbas, 2023). These disparities underscore the potential of tailored diets incorporating natural ingredients to enhance the reproductive success of aquatic

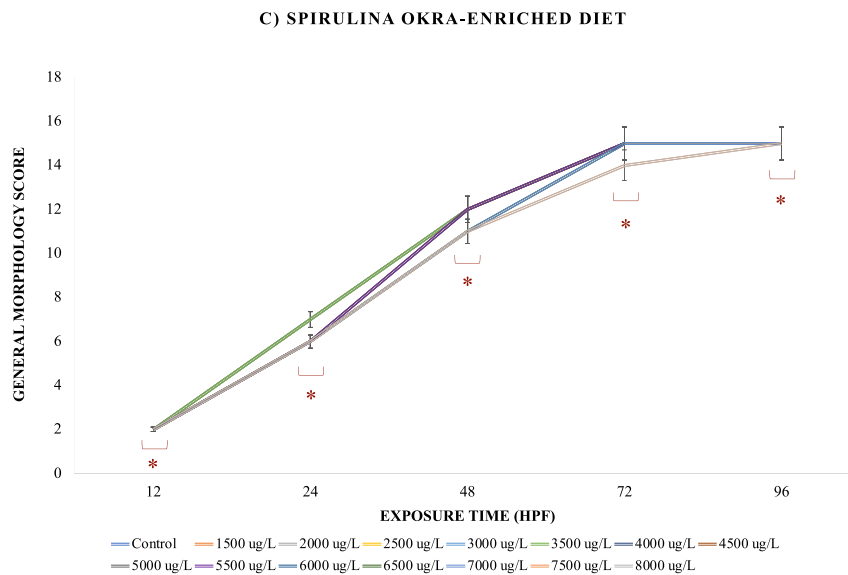


Fig. 3. (continued).

organisms like zebrafish (Amenyogbe, 2023). However, further research is crucial to gain a deeper understanding of the mechanisms underlying the observed differences in oocyte viability among these dietary regimens, presenting exciting possibilities for improved aquaculture practices and environmental conservation.

Exposure to aluminum resulted in various alterations in *D. rerio* embryos, including pericardial edema, craniofacial malformations, growth retardation, and other abnormalities. Notably, embryos chronically exposed to a spirulina-enriched diet exhibited a significant reduction in aluminum-induced malformations. Furthermore, embryos from the spirulina-okra diet group displayed even fewer malformations when compared to those fed the spirulina-enriched diet.

The mechanisms through which spirulina-enriched and spirulina-okra-enriched diets protect against aluminum-induced embryotoxicity likely involve several interconnected factors: (1) *Antioxidant defense*: both spirulina and okra are rich in antioxidants, including vitamins (e.g., C and E) and phytochemicals (e.g., polyphenols and carotenoids) (Ali and Bhattacharjee, 2023; Lakhdar et al., 2023; Seğmen and Özdamar Ünlü, 2023). These antioxidants can scavenge reactive oxygen species (ROS) generated by aluminum exposure, reducing oxidative stress and protecting developing embryos (Ijarotimi et al., 2023; Jha et al., 2023; Parmar et al., 2023), (2) *metal chelation*: Okra is known to contain compounds with metal-chelating properties (Dhiman et al., 2023; Saleh and Fadillah, 2023). These compounds can bind to aluminum ions, reducing their bioavailability and preventing their toxic effects on embryonic development; (3) *anti-inflammatory effects*: Inflammation is a common response to aluminum exposure, and chronic inflammation can harm embryonic development (Logan et al., 2023). Spirulina, in particular, has demonstrated anti-inflammatory properties (Saraswathi and Kavitha, 2023), reducing inflammation and mitigating the detrimental effects of aluminum on embryogenesis (Abdelghany et al., 2023; Kumar et al., 2023); (4) *nutrient density*: Spirulina is renowned for its high nutritional value, containing essential amino acids, vitamins, and minerals (Terzioğlu et al., 2023). Okra also contributes essential nutrients, such as dietary fiber, vitamins (e.g., A and C), and minerals (e.g., potassium and calcium) (Chadha, 2023). A diet enriched in spirulina provides a rich source of nutrients crucial for normal development, potentially counteracting the negative impacts of aluminum; (5) *synergistic effects*: The unique combination of antioxidants, metal-chelating compounds, and anti-inflammatory agents from both spirulina and okra may enhance the overall protective mechanisms against aluminum-induced embryotoxicity; (6) *detoxification*: Okra's mucilaginous

properties may aid in the removal of toxins, including aluminum, from the digestive tract (Pourakbar et al., 2023; Ormanli et al., 2023). This could reduce aluminum absorption, limiting its availability to interfere with embryonic development; (7) *enhanced immune function*: Spirulina has been reported to modulate immune responses (El-Shall et al., 2023; Iatrou et al., 2023), potentially strengthening the embryo's ability to defend against the toxic effects of aluminum.

In short, spirulina-enriched diets and spirulina-okra-enriched diets are likely effective in protecting against aluminum-induced embryotoxicity by combining antioxidant defenses, metal chelation, anti-inflammatory effects, nutrient density, synergistic interactions, detoxification, and potentially improved immune function. These mechanisms collectively reduce the number of malformations in zebrafish embryos compared to those fed with a commercial diet, which may lack these specific protective attributes.

Diets enriched with spirulina and spirulina-okra mixture likely reduce oxidative stress in zebrafish embryos through a combination of antioxidant compounds and the activation of endogenous antioxidant defense mechanisms. Spirulina is renowned for its high levels of antioxidants, including vitamins C and E, beta-carotene, and phycocyanin (Ajayi, 2023; L. Wang et al., 2023; Y.Y. Wang et al., 2023). These antioxidants have the capability to neutralize reactive oxygen species (ROS) and free radicals generated during exposure to aluminum, thereby preventing cellular damage and oxidative stress (Ansari et al., 2023; Mohan et al., 2023; Parveen et al., 2023).

The inclusion of okra in the diet complements the antioxidant arsenal. Okra contains polyphenols, flavonoids, and other phytochemicals that exhibit antioxidant properties. When combined with spirulina, these antioxidants work synergistically to enhance the overall antioxidant capacity of the diet. This cooperative action strengthens the embryo's ability to counteract oxidative stress by intercepting and neutralizing ROS effectively (Pruteanu et al., 2023).

Furthermore, these innovative diets may trigger the upregulation of endogenous antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Younis et al., 2023; Parmar et al., 2023). These enzymes play crucial roles in detoxifying ROS and maintaining cellular redox balance (Jena et al., 2023; Jomova et al., 2023; Sachdev et al., 2023; Zhu et al., 2023). Their increased activity under the influence of spirulina and okra-enriched diets can further contribute to reducing oxidative stress in zebrafish embryos. This dual mechanism, involving both direct antioxidant compounds and the stimulation of intrinsic antioxidant defenses, collectively promotes a healthier cellular

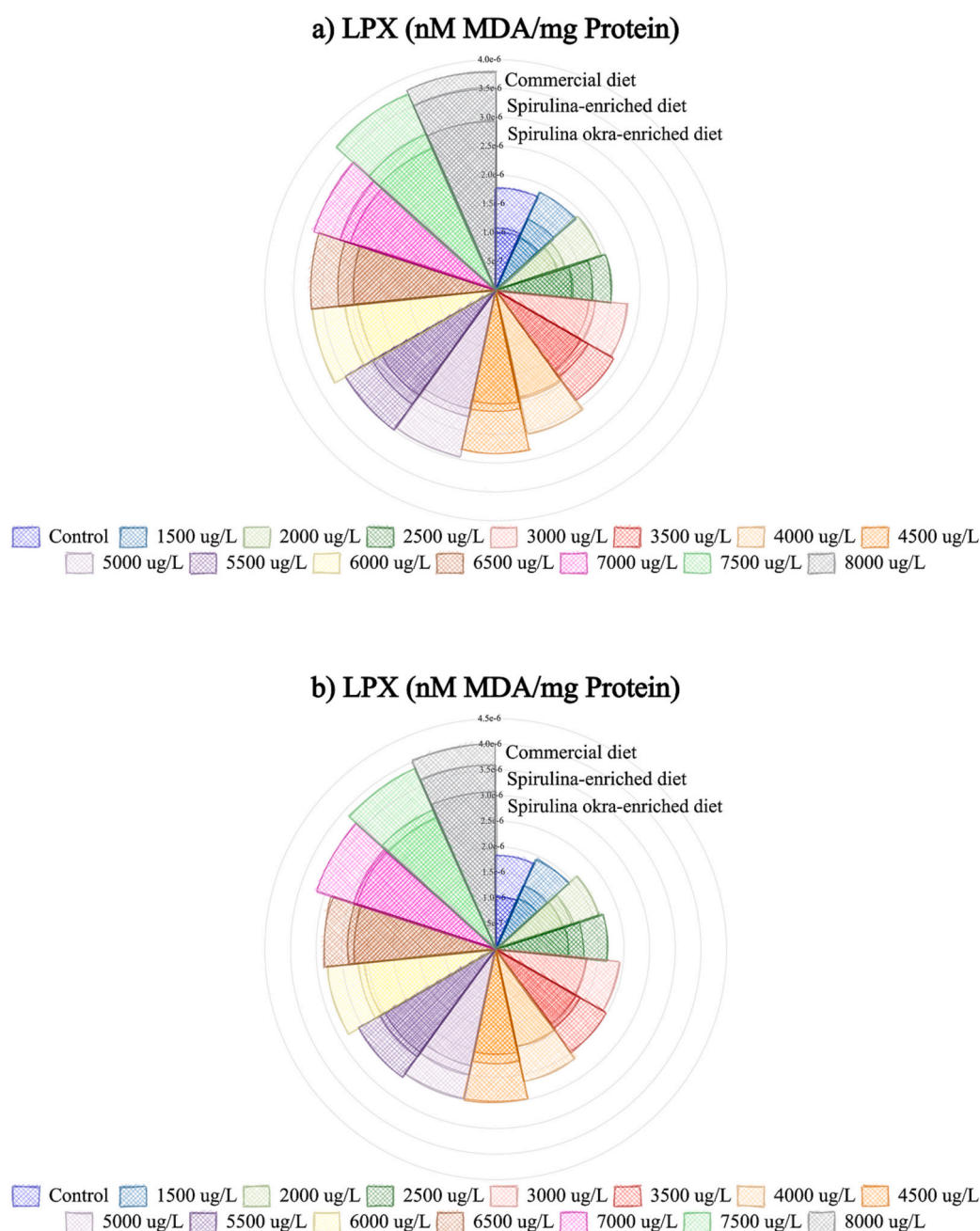


Fig. 4. Oxidative damage response [a) LPX at 72 hpf, b) LPX at 96 hpf, c) HPX at 72 hpf, d) HPX at 96 hpf, e) POX at 72 hpf and f) POX at 96 hpf] of *D. rerio* after acute exposure to AlCl_3 . Data represent mean $\pm$ standard deviation (SD) of three independent experiments and three replicates per experiment ($n = 9$). MDA stands for Malondialdehyde. CHP stands for cumene hydroperoxide. CO stands for reactive carbonyls ($\text{C} = \text{O}$). At 72 hpf: LPX $F(44,360) = 159.9$; $n = 9$; $p < 0.001$; HPX $F(44,360) = 220.2$; $n = 9$; $p < 0.001$; POX $F(44,360) = 49.9$; $n = 9$; $p < 0.001$. At 96 hpf LPX $F(44,360) = 170.7$; $n = 9$; $p < 0.001$; HPX $F(44,360) = 227.1$; $n = 9$; $p < 0.001$; POX $F(44,360) = 51.34980$; $n = 9$; $p < 0.001$.

environment, lowers oxidative stress levels, and ultimately leads to a reduced incidence of malformations in zebrafish embryos exposed to aluminum.

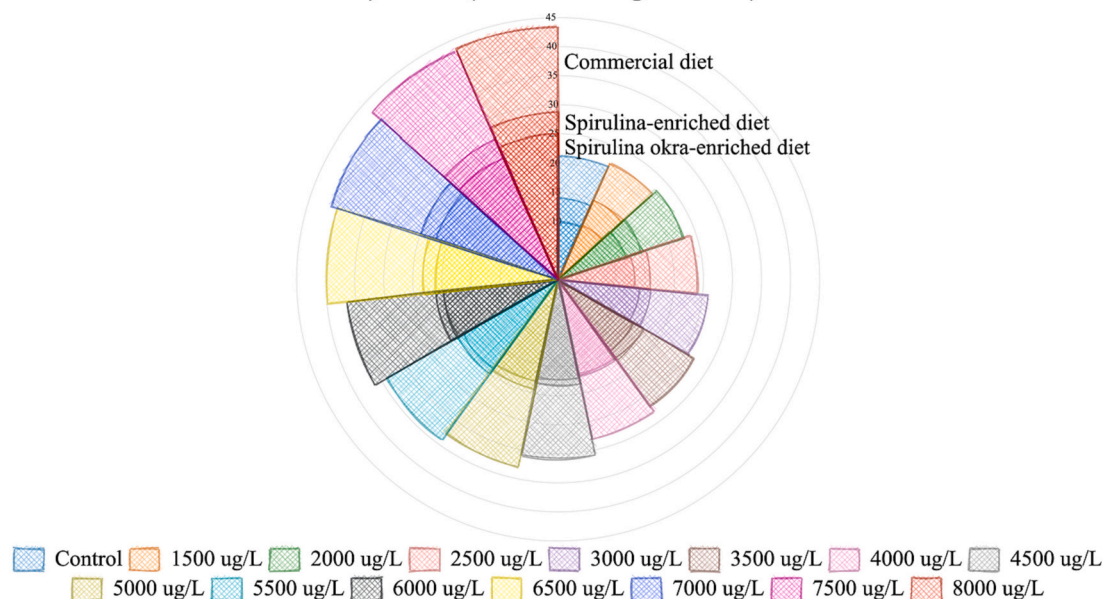
The influence of spirulina and okra-spirulina enriched diets on the up-regulation of *nrf1* and *nrf2* genes, as well as the down-regulation of *p53*, *bax*, and *bcl2* genes, suggests a multi-faceted mechanism through which these diets may protect against aluminum-induced embryotoxicity and reduce malformations in *D. rerio* (zebrafish) embryos.

Activation of antioxidant pathways (*nrf1* and *nrf2*): *Nrf1* and *Nrf2* are transcription factors known to regulate the expression of antioxidant

genes (Cao et al., 2023; Elizalde-Velázquez et al., 2023; Kanwugu and Glukhareva, 2023). Up-regulation of these genes indicates an enhanced antioxidant defense system in response to the diets. Spirulina and okra-spirulina are rich in antioxidants, including vitamins and phytochemicals, which can activate these pathways (Tossetta et al., 2023; Tuong et al., 2023). This increased antioxidant activity can counteract the oxidative stress induced by aluminum exposure, reducing cellular damage and malformations.

Suppression of pro-apoptotic genes (*p53* and *bax*): The down-regulation of *p53* and *bax* genes suggests a potential anti-apoptotic effect of the diets. Aluminum exposure can trigger apoptosis (cell

c) HPX (uM CHP/mg Protein)



d) HPX (uM CHP/mg Protein)

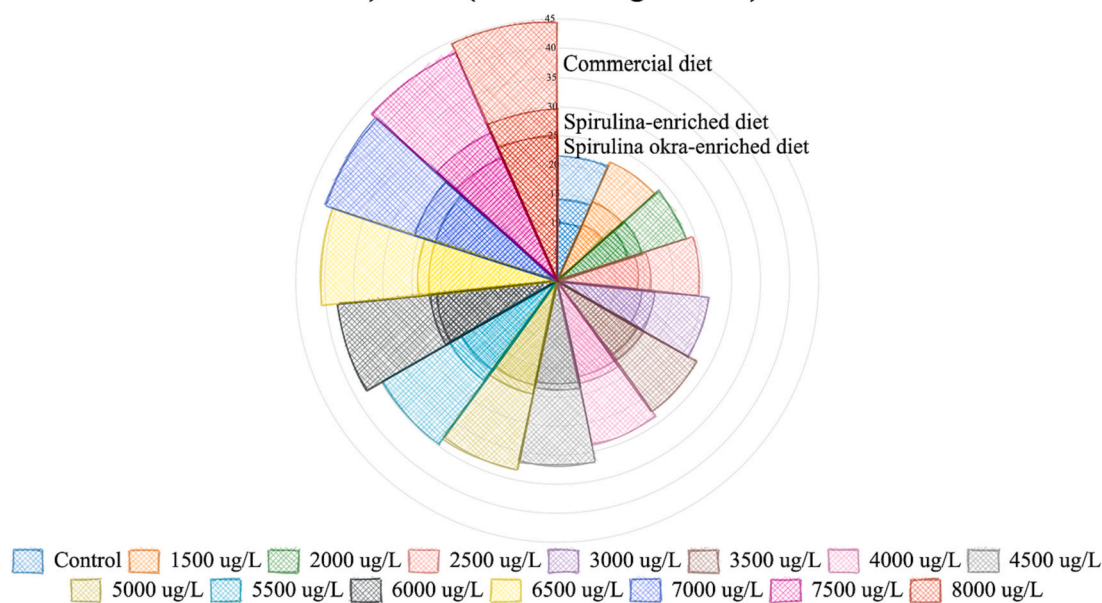


Fig. 4. (continued).

death) through pathways involving these genes (Arini et al., 2023; Elganzoury et al., 2023). By inhibiting their expression, spirulina and okra-spirulina diets may prevent excessive cell death in developing embryos. This preservation of cells can contribute to normal embryonic development and fewer malformations.

Enhanced cell survival (*bcl2*): The down-regulation of *bcl2* indicates reduced expression of this anti-apoptotic gene (Yu et al., 2023). While this might seem counterintuitive, it can be beneficial in the context of embryonic development. Lower *bcl2* expression can promote programmed cell death (apoptosis) in cells that are damaged or malformed due to aluminum exposure (Kumar et al., 2023; Peng et al., 2023). This selective apoptosis can help eliminate abnormal

cells, allowing healthy cells to thrive and reducing the overall burden of malformations.

In summary, the influence of spirulina and okra-spirulina enriched diets on these gene expression patterns suggests a coordinated response to mitigate the harmful effects of aluminum on embryonic development. Up-regulation of antioxidant genes (*nrf1* and *nrf2*) enhances the cellular defense against oxidative stress, while down-regulation of pro-apoptotic genes (*p53* and *bax*) and moderate down-regulation of the anti-apoptotic gene (*bcl2*) collectively contribute to reducing cell damage, promoting cell survival, and ultimately decreasing the number of malformations in zebrafish embryos exposed to aluminum. These findings underscore the potential of these dietary interventions as protective strategies against

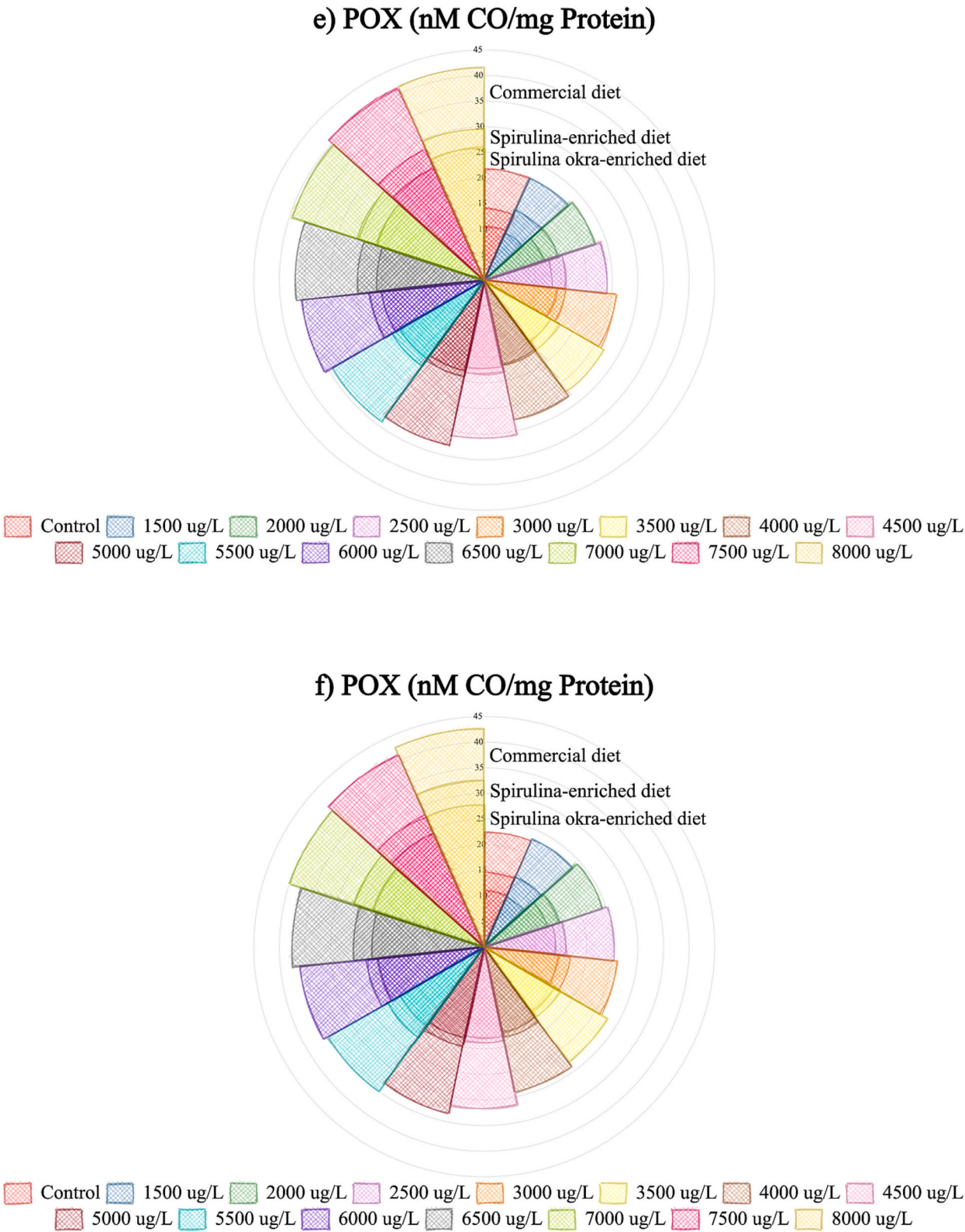
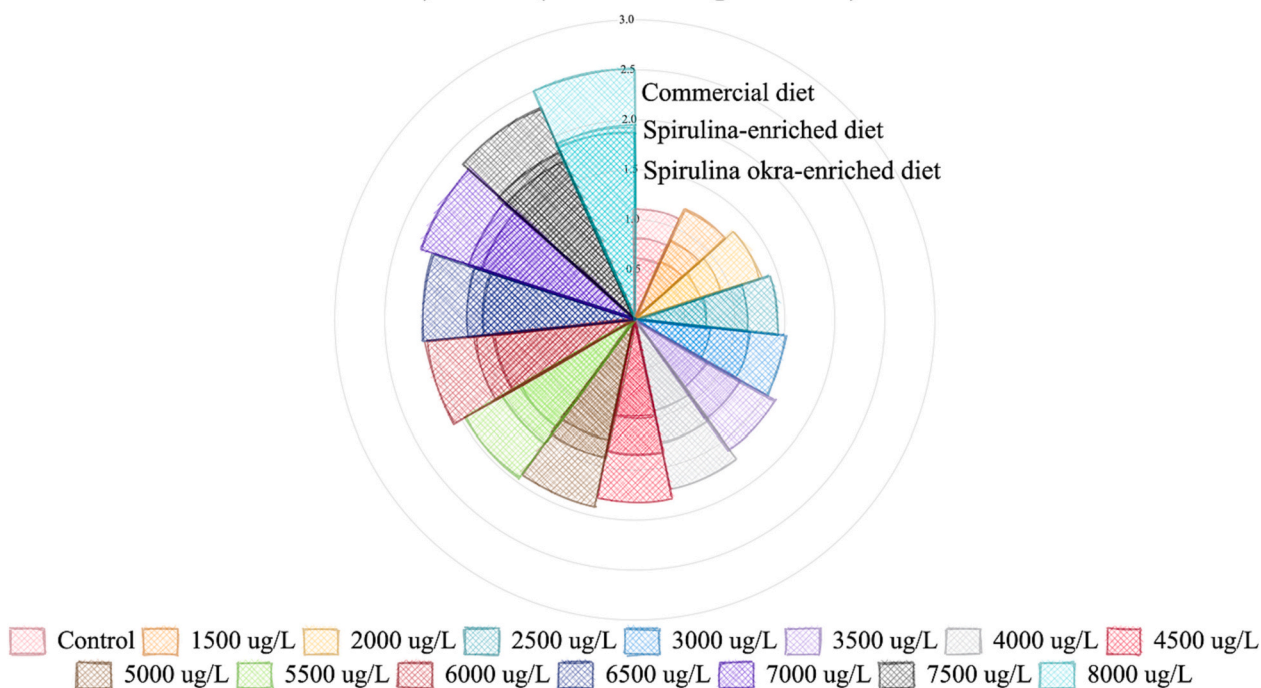


Fig. 4. (continued).

aluminum-induced embryotoxicity. As we move forward, it is essential to delve deeper into the underlying molecular mechanisms that drive these protective effects and explore potential long-term consequences on fish populations and food safety. Ultimately, this study underscores the importance of considering innovative dietary approaches in environmental conservation strategies

and the safeguarding of aquatic ecosystems from the perils of aluminum contamination. The comparison of the results of this study with other relevant studies in this field is fundamental to contextualize the importance of this research. Several studies have explored the impact of dietary interventions on aquatic organisms and their responses to environmental

a) SOD (IU SOD/mg Protein)



b) SOD (IU SOD/mg Protein)

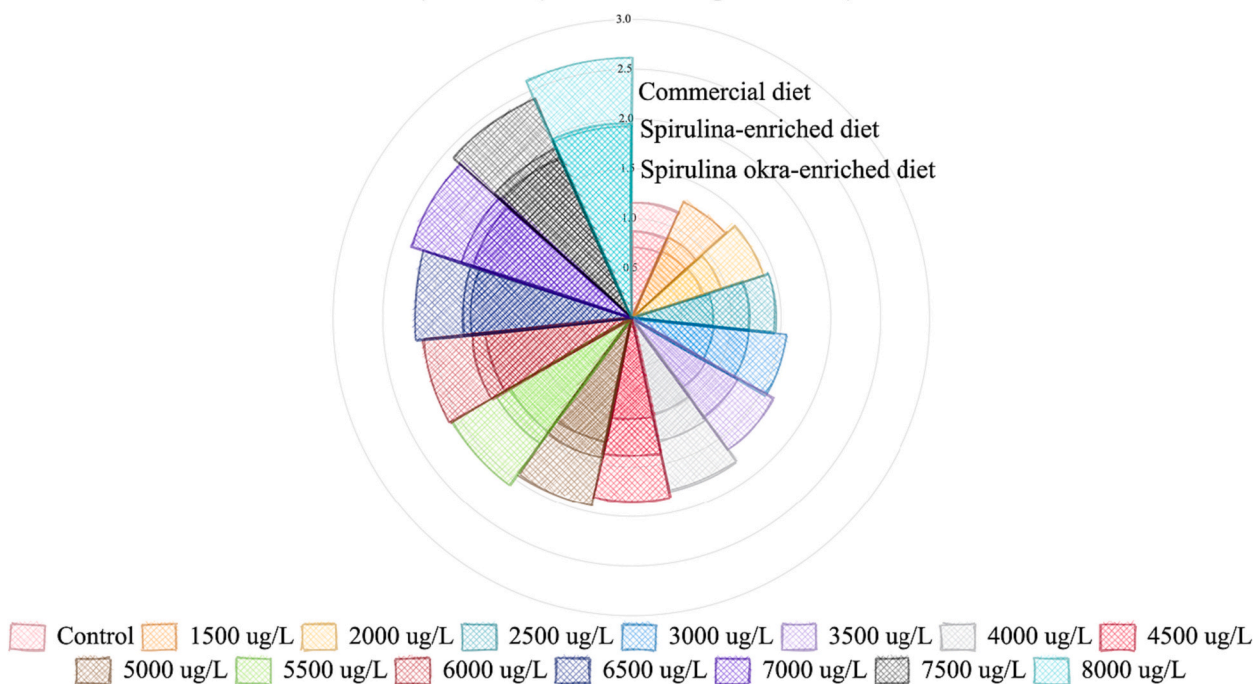


Fig. 5. Antioxidant response [a) SOD at 72 hpf, b) SOD at 96 hpf, c) CAT at 72 hpf and d) CAT at 96 hpf] of *D. rerio* after acute exposure to AlCl_3 . Data represent mean $\pm$ standard deviation (SD) of three independent experiments and three replicas per experiment ($n = 9$). At 72 hpf SOD $F(44,360) = 71.7$; $n = 9$; $p < 0.001$; CAT $F(44,360) = 1536.2$; $n = 9$; $p < 0.001$. At 96 hpf SOD $F(44,360) = 87.5$; $n = 9$; $p < 0.001$; CAT $F(44,360) = 1545.8$; $n = 9$; $p < 0.001$.

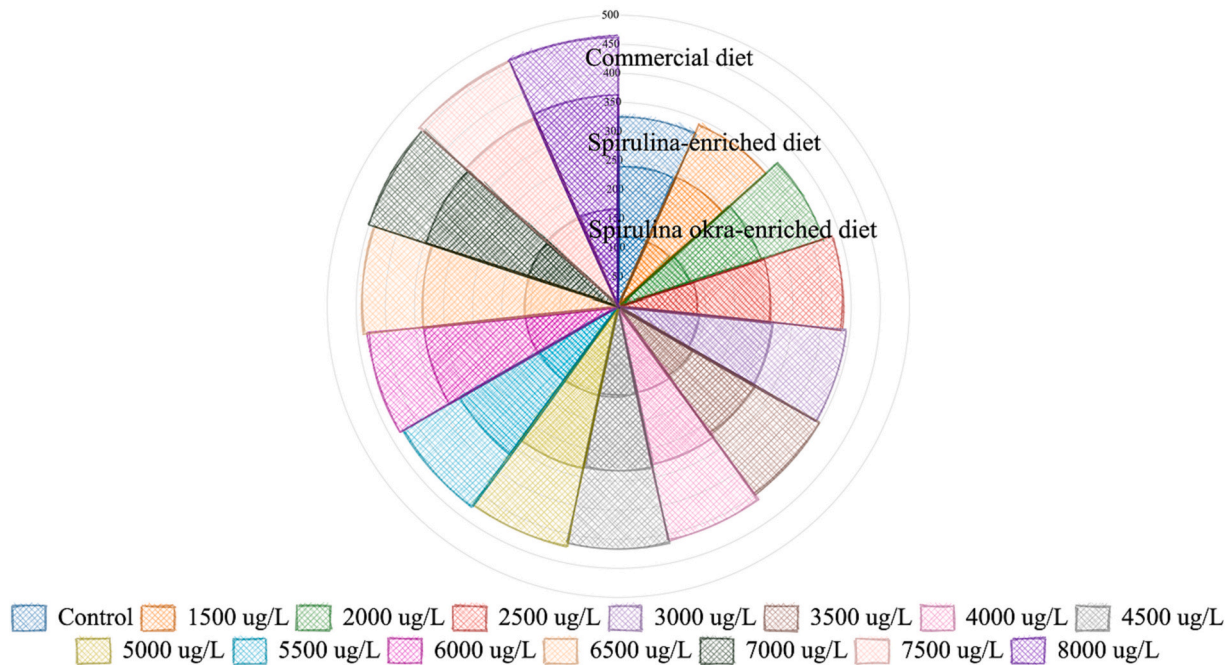
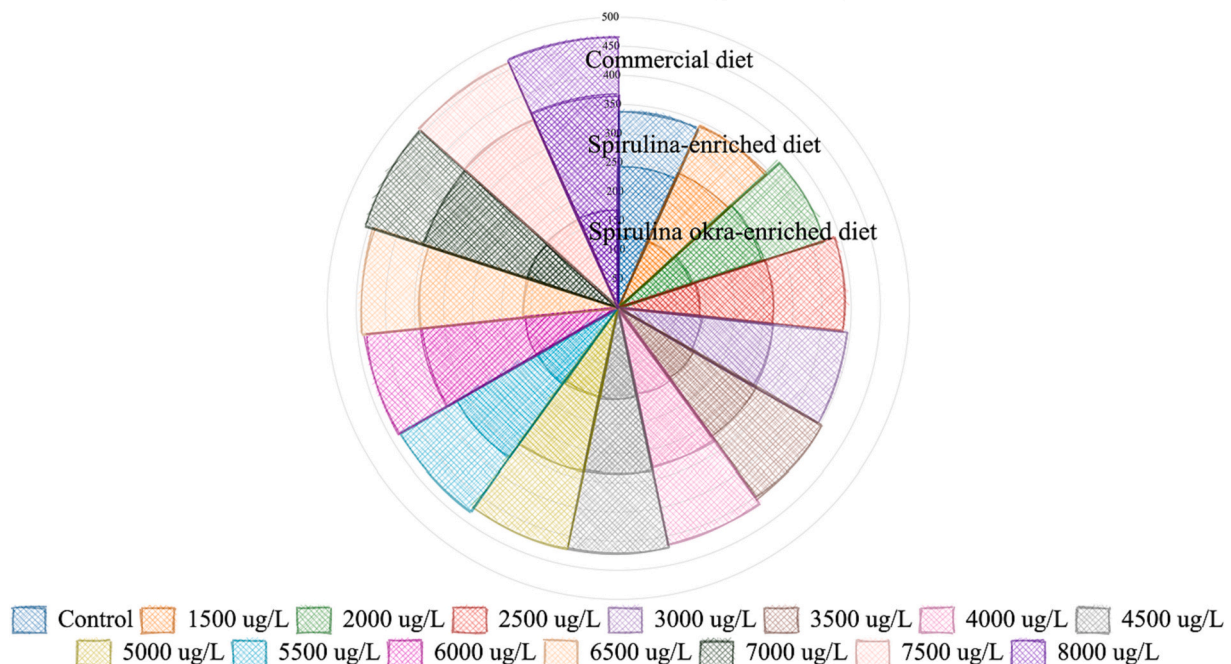
c) CAT (mM H₂O₂/mg Protein)d) CAT (mM H₂O₂/mg Protein)

Fig. 5. (continued).

stressors. While some research has focused on the benefits of individual dietary components such as spirulina or okra, this study uniquely combines these ingredients, revealing a synergistic effect that significantly outperforms the commercial diet in reducing aluminum-induced embryotoxicity and malformations. Research by [Amenyogbe \(2023\)](#) has demonstrated the potential of custom-tailored diets enriched with natural ingredients in enhancing the reproductive success of aquatic organisms, aligning with our conclusions. Additionally, studies by

[Ijarotimi et al. \(2023\)](#) and [Pruteanu et al. \(2023\)](#) have highlighted the antioxidant properties of spirulina and the potential of okra in metal chelation and reducing oxidative stress, which aligns with the proposed mechanisms. The research extends this knowledge by providing a comprehensive evaluation of diet-enriched strategies, elucidating the protective effects against aluminum toxicity. The potential of natural dietary strategies to mitigate the detrimental effects of environmental pollutants such as Al should be further explored to elucidate the

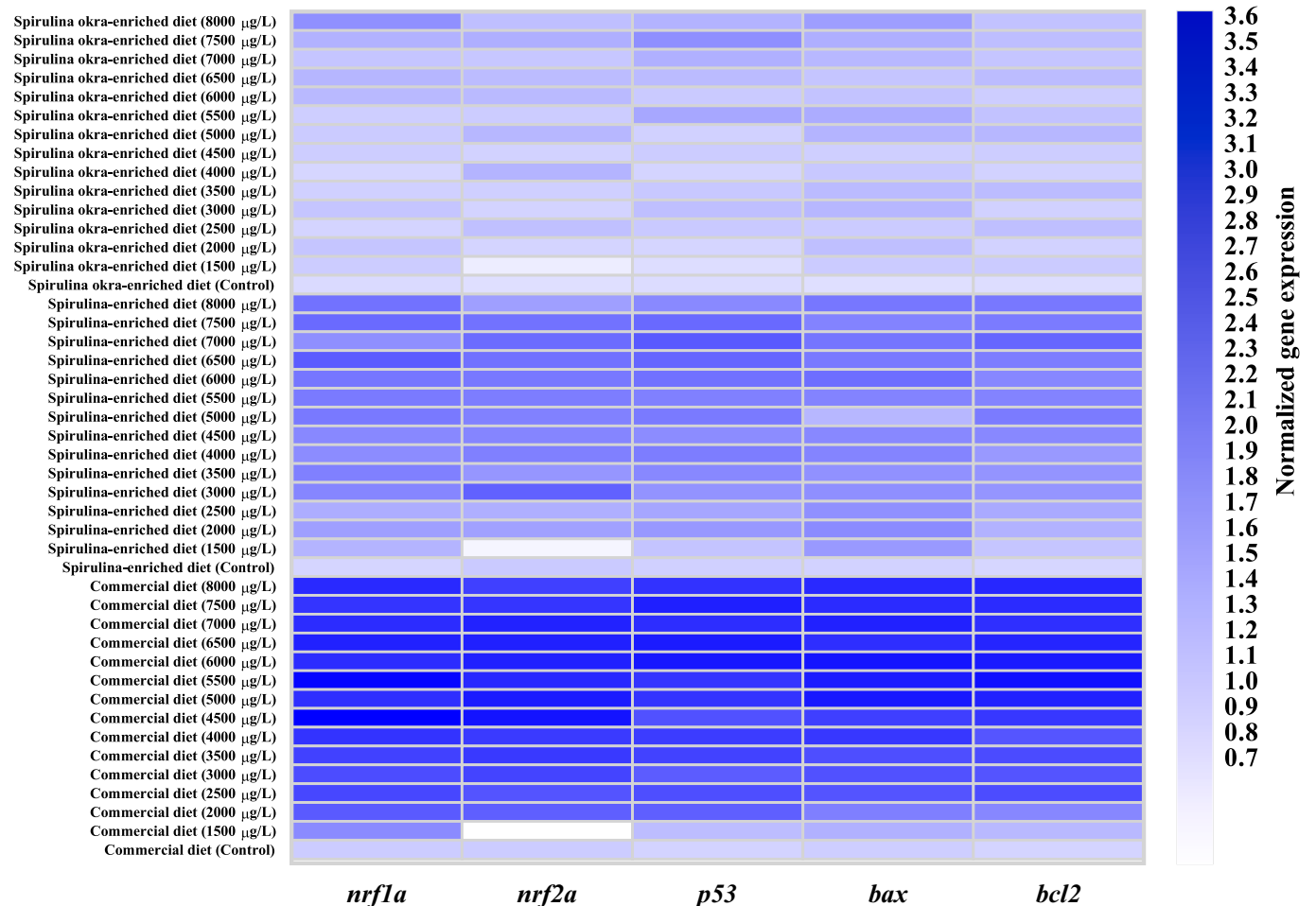


Fig. 6. Acute exposure to AlCl₃ alters different genes related to oxidative stress (*nrf1a* and *nrf2*) and apoptosis (*p53*, *bax*, and *bcl2*) in the zebrafish embryos. Data represent mean ± standard deviation (SD) of three independent experiments and three replicas per experiment (n = 9). *nrf1a*: nuclear factor erythroid 2-related factor 1a, *nrf2*: nuclear factor erythroid 2-related factor 2a, *bax*: BCL2 associated X, *p53*: tumor protein p53, *bcl2*: BCL2 apoptosis regulator. Statistical values: *nrf1* F (44,360) = 2.774; n = 9; p = 1.0, *nrf2* F (44,360) = 12.84; n = 9; p = 0.997, *p53* F (44,360) = 2.266; n = 9; p = 1.0, *bax* F (44,360) = 2.021; n = 9; p = 1.0, *bcl2* F (44,360) = 2.751; n = 9; p = 1.0.

mechanisms that favor toxicity mitigation.

5. Conclusion

In conclusion, this comparative study investigating the mitigation of aluminum-induced embryotoxic effects in *D. rerio* using spirulina and okra-spirulina diets has unveiled promising insights into enhancing the reproductive health and well-being of aquatic organisms. Our findings have illuminated several key aspects of these innovative dietary interventions: (1) Enhanced oocyte viability: the spirulina-okra-enriched diet exhibited a remarkable capacity to stimulate higher oocyte viability compared to both the spirulina diet and the commercial diet. This result underscores the potential of tailored diets enriched with specific natural ingredients, offering a promising avenue for enhancing reproductive success in aquatic organisms; (2) reduced embryotoxicity: Exposure to aluminum induced several malformations in *D. rerio* embryos. However, the spirulina-enriched diet and, even more so, the spirulina-okra diet significantly alleviated aluminum-induced embryotoxicity. This reduction in malformations highlights the protective efficacy of these dietary regimens against the harmful effects of aluminum; (3) mitigated oxidative stress: the mechanisms underlying the protection against aluminum-induced embryotoxicity likely involve a multifaceted approach. Both spirulina and okra contribute to the antioxidant defense system, reducing oxidative stress generated by aluminum exposure. This reduction in oxidative stress is critical for maintaining healthy cellular

environments and preventing malformations; (4) modulation of gene expression: the diets enriched with spirulina and okra-spirulina blends influenced the expression of key genes. Up-regulation of antioxidant genes (*nrf1* and *nrf2*) and down-regulation of pro-apoptotic genes (*p53* and *bax*) collectively contribute to reducing cellular damage and promoting cell survival. These gene expression patterns reflect a coordinated response to mitigate the harmful effects of aluminum on embryonic development.

In light of these findings, it is evident that spirulina and okra-spirulina-enriched diets hold great promise as effective strategies for mitigating aluminum-induced embryotoxicity in *D. rerio*. These dietary interventions offer a holistic approach by enhancing reproductive success, reducing embryotoxicity, alleviating oxidative stress, and modulating gene expression to promote healthier and more resilient aquatic ecosystems.

This research not only deepens our understanding of aluminum toxicity but also underscores the potential of natural dietary interventions in safeguarding the health of aquatic organisms and, by extension, the safety of our food chain. As we continue to grapple with environmental challenges, such innovative approaches can play a pivotal role in fostering sustainability and resilience in aquatic ecosystems. Further research and exploration of these dietary interventions are warranted to unlock their full potential in environmental conservation and aquaculture practices.

CRediT authorship contribution statement

Conceptualization: Erika Mariana García-Avalos & Leobardo Manuel Gómez-Oliván; Data curation: Erika Mariana García-Avalos; Formal analysis: Leobardo Manuel Gómez-Oliván; Methodology: Erika Mariana García-Avalos, María Dolores Hernández-Navarro & Karina Elisa Rosales-Pérez; Project administration: Leobardo Manuel Gómez-Oliván; Software: Muhammad Hamzah Saleem & Nely San Juan-Reyes; Validation: Nely San Juan-Reyes & Gustavo Axel Elizalde-Velázquez; Visualization: Nely San Juan-Reyes; Writing—original draft: Leobardo Manuel Gómez-Oliván, Muhammad Hamzah Saleem & Gustavo Axel Elizalde-Velázquez; Writing—review and editing: Leobardo Manuel Gómez-Oliván & Muhammad Hamzah Saleem. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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