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Research article

Chronic toxicity of antibiotics and global warming in *Danio rerio*: Biomarker responses and toxicological effects

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ABSTRACT

The combined influence of multiple stress factors on natural ecosystems is a critical concern, as neglecting their effects could compromise essential biological functions. However, limited studies have explored the combined effects of antibiotics and global warming on aquatic ecosystems, leaving a gap in understanding their interaction. This study aimed to assess the toxicity of environmentally relevant concentrations of sulfamethoxazole (SMX: 150 μg/L), trimethoprim (TRIM: 30 μg/L), and their mixture (MIX: 150 μg SMX/L + 30 μg TRIM/L) on *Danio rerio* at three temperature conditions: standard (26 °C), moderately high (28 °C), and high (32 °C) temperatures. A multi-biomarker approach was used to evaluate the organism's biological status (e.g., antioxidant/detoxification defense enzymes, lipid peroxidation, cholinergic neurotransmission, energetic metabolism, DNA damage). Results indicated that rising temperatures influenced the toxicity level of each antibiotic differently to *D. rerio*. At 26 °C, all the antibiotics were marginally toxic, and major alterations were observed (oxidative stress and neurotoxicity). Increasing temperature to 28 °C, the toxicity increased, with SMX and MIX exhibiting moderate toxicity, and severe alterations (neurotoxicity and DNA damage). In contrast, TRIM showed only slight toxicity and recorded negligible alterations (antioxidant defense alterations). At higher temperature (32 °C) individual antibiotics revealed slightly toxic with negligible alterations. However, MIX at 32 °C was more toxic, and severe damage was observed (e.g., higher DNA damage). These findings reveal a pressing and alarming threat: combined contaminants impact and climate change could drive aquatic ecosystems toward collapse. Understanding how these stressors interact is critical to preventing potentially irreversible damage to aquatic life.

1. Introduction

In recent years, there has been a growing global concern about the impact of environmental contaminants on ecosystems and human health (Noyes et al., 2009). Intensifying this problem, the effects of climate change, including rising temperatures, further stress ecosystems, creating complex interactions that can amplify the toxicity of environmental contaminants (Bethke et al., 2023; Noyes et al., 2009). In recognition of these challenges, the 2030 Agenda for Sustainable Development identifies climate change as "one of the greatest ecosystem challenges of this century" (United Nations, 2021). Within objective 13, which aims to "take urgent action to combat climate change and its impact," member states acknowledge that rising global temperatures can have severe consequences for vulnerable regions, particularly those of poorer and underdeveloped nations (United Nations, 2023a). Furthermore, the availability of basic needs (freshwater, food security, and energy) is threatened, putting at risk the survival of "societies and biological support systems of the planet" (United Nations, 2023b). As reported by the IPCC's Sixth Assessment Report, the climate is warming rapidly, having recorded global surface temperatures reaching 1.1 °C above 1850–1900 in 2021–2020 (IPCC, 2023). According to the projections based on different greenhouse gas emission scenarios, the average global surface temperature is expected to rise by 1.0–1.8 °C up to 3.3–5.7 °C by 2081–2100 (IPCC, 2023). Considering the worst-case

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projection scenario, an increase of 4 °C of the global surface temperature can represent a very high risk/impact on freshwater ecosystems, causing biodiversity loss and structural change (IPCC, 2023).

Temperature has a significant impact on the health and functioning of organisms, impacting all levels of biological organization, from cellular biochemical processes to population growth (Adamczuk, 2024; Noyes et al., 2009). This is one of the climate change-related factors that profoundly change freshwater ecosystems, increase organism vulnerability, and reduce their ability to adapt to new conditions (Sanpradit et al., 2024). Several authors have already reported that the physiology and metabolic alterations of freshwater organisms (e.g., *Daphnia magna* and *Danio rerio*) are highly influenced by temperature (e.g., metabolic and immune alterations, oxidative stress) (Jannat et al., 2024; Kazmi et al., 2022; Sanpradit et al., 2024). However, natural ecosystems are exposed to multiple stressors, and the combination of these stresses should not be neglected as it may compromise existing biological support systems.

Beyond the issue of global warming, other critical environmental concerns have garnered global attention. Notably, one such challenge is the excessive use of pharmaceuticals, particularly antibiotics, which pose significant risks to ecosystems (Cars and Jasovsky, 2015). The number of antibiotics used for the treatment/prevention of multiple infections and their residues has increased exponentially worldwide (e.g., an increase of 16.4 to 20.0 defined daily doses per 1000 inhabitants per day in Europe between 2020 and 2023) (ECDE, 2024). This increase represents a potential risk for terrestrial and aquatic ecosystems [due to their biological activity (Grinten et al., 2010)] once they have been detected in low concentrations (ng/L or μg/L) in surface and groundwaters (Carvalho and Santos, 2016; Chen et al., 2021; Diogo et al., 2023b). Two of the most used antibiotics in the last 50 years are sulfamethoxazole (SMX) and trimethoprim (TRIM) which led to their inclusion in the 3rd Watch List of substances to monitor under the Water Framework Directive to assess the ecological status of aquatic ecosystems (Cortes et al., 2020; Cortes et al., 2022). These antibiotics, as well as their mixture (MIX), are applied in human and animal medicine, aquaculture, and agriculture (Carvalho and Santos, 2016; Ho and Juurlink, 2011), and are commonly detected in freshwater ecosystems (Chen et al., 2021).

Considering that the bioavailability and toxicity of antibiotics increase in response to rising temperatures (Danner et al., 2021; Kazmi et al., 2022; Wiles et al., 2020), the study of the combined effects of these factors is essential. It has already been described that the temperature has a significant impact on the environmental fate, distribution, and toxicity of antibiotics (Hutton et al., 2024; Kazmi et al., 2022). By relating the antibiotics effects (such as SMX and TRIM) to climate change scenarios, it is possible to gain insights into the challenges and risks that freshwater organisms may face. It is not only public and environmental health issues (preserving the biodiversity of ecosystems), but it is also critical to global development progress. Recently, studies reported that SMX, TRIM, and their MIX affected individually and sub-individually different freshwater organisms (e.g., *Scenedesmus obliquus, Lemna minor, Daphnia magna*, and *Danio rerio*) (Diogo et al., 2024; Xiong et al., 2019); however, no studies have been found exploring the combined effects of environmentally relevant concentrations of these antibiotics and global warming predictions. Thus, to understand environmental threats on freshwater ecosystems due to antibiotic contamination and global warming simultaneously, this study aims to assess the effects of environmentally relevant concentrations of SMX, TRIM, and MIX on fish *D. rerio* evaluating the biological health status across different ecologically relevant warming scenarios (26, 28, and 32 °C), utilizing a multi-biomarker approach. This study provides novel insights into the underexplored interaction between antibiotics and climate warming by evaluating the temperature-dependent toxicological effects of environmentally relevant concentrations of SMX, TRIM, and their mixture on *Danio rerio*. Through a multi-biomarker approach, the research provides an integrated assessment of potential synergistic impacts on aquatic health. By aligning potential environmental conditions with climate projections, the study contributes to a more realistic understanding of ecological risks, supporting efforts to protect biodiversity, safeguard water quality, and inform global environmental management strategies.

2. Material and methods

2.1. Test organism: Danio rerio

Danio rerio (zebrafish) is a freshwater fish widely used as a standard organism for ecotoxicological studies (OECD, 2000). To conduct this study, the juveniles used in the experiment were born from a laboratory broodstock and reared under standard laboratory conditions in a zebrafish facility at CIIMAR - Interdisciplinary Centre of Marine and Environmental Research (Matosinhos, Portugal), until transferred to the experimental room for acclimation. The quarantine/acclimation period (three weeks) was conducted in 60 L tanks with continuous aerated and dechlorinated tap water, with controlled conditions of photoperiod (16 h^L:8 h^D) and temperature (26 ± 1 °C). Every two days, during the quarantine period, water quality parameters (temperature, conductivity, pH, dissolved oxygen, ammonium, and nitrite levels) were monitored, and the organisms were fed ad libitum with commercial zebrafish food (Zebrafeed 400–600 μm by Sparos). Organisms were considered proper/healthy for the assays since no disease signals or death were recorded, at least for 15 days. Trained researchers (following FELASA category C recommendations) directed the experiment, and all procedures were conducted according to the recommendations of the European Union Directive (2010/63/EU) while operating under the Portuguese Law (DL 113/2013) on the protection of animals for scientific purposes (Ministério da Agricultura, 2013). The experimental protocol was approved by the Animal Welfare and Ethics Body committee of the Interdisciplinary Centre of Marine and Environmental Research (ORBEA-CIIMAR).

2.2. Chemicals and stock solutions

Sulfamethoxazole (SMX; molecular weight 253.28 g/mol; ≥ 98.0 % purity; CAS: 723–46-6) and trimethoprim (TRIM; molecular weight 290.3 g/mol; ≥ 98.5 % purity; CAS: 738–70-5) were acquired from Sigma Aldrich. SMX and TRIM stock solutions (100 and 50 mg/L, respectively) were prepared by diluting each antibiotic in dechlorinated tap water. The nominal concentrations tested (150 μg SMX/L and 30 μg TRIM/L), were chosen based on the maximum concentrations detected in surface water reported by the literature (Kairigo et al., 2020; Khan et al., 2013). The effects of a mixture of SMX and TRIM (MIX = 150 μg SMX/L + 30 μg TRIM/L) were also evaluated to simulate real environmental conditions (Carvalho and Santos, 2016; Kairigo et al., 2020; Khan et al., 2013).

2.3. Chronic assay

The chronic assay was carried out under laboratory-controlled conditions similar to those adopted during the acclimation period, and according to OECD test guideline n° 215 (OECD, 2000). Juvenile individuals of *D. rerio* (2 months old; 1.43 ± 0.01 cm; 0.041 ± 0.001 g) were exposed for 28 days to different antibiotics treatments: SMX (150 μg/L), TRIM (30 μg/L), MIX (150 μg SMX/L + 30 μg TRIM/L), and a control group (CTL; without antibiotics). Each treatment was tested at three temperature scenarios: standard temperature (26 °C), moderately high temperature (28 °C), and high temperature (32 °C), selected by increasing the standard temperature for *D. rerio* (26 °C) by 2 °C and 6 °C, according to global warming projections from the Intergovernmental Panel on Climate Change and Climate Action Tracker (IPCC, 2023; Stockwell et al., 2021). Projections based on different greenhouse gas emission scenarios indicate that the average global surface temperature

is expected to rise by 1.0–1.8 °C (max ~2 °C; low emissions scenario) up to 3.3–5.7 °C (max ~6 °C; high emissions scenario) by 2081–2100 (IPCC, 2023). For freshwater ecosystems, projections indicate that surface water temperatures are likely to increase significantly. By 2081–2099, the average surface temperatures in 46,557 European lakes are expected to rise by 2.9 °C, 4.5 °C and 6.5 °C, depending on the scenario, compared to the historical baseline of 1981–1999 (IPCC, 2023; Woolway et al., 2020).

Fish were distributed in thirty-six 2-L glass aquaria (randomly distributed in the exposure room), with three replicates per treatment (3 aquaria per treatment, each one with 6 fish). Fish were fed, and ~ 80 % of the medium was renewed every 48 h. According to guideline OECD n° 215 (OECD, 2000), physical and chemical water parameters (pH, temperature, conductivity, and dissolved oxygen) were measured twice a week, using a multiparametric probe (Multi 3630 IDS SET F), to validate the water quality during the assay. A bench photometer (Spectroquant Multy Colimeter) was used to quantify ammonium and nitrites after collecting water aliquots from all aquaria (before medium renewal).

2.4. Antibiotic quantifications

For the quantification of SMX, TRIM, and MIX analytical concentrations (Table 1), a volume of 50 mL of water was randomly collected from a replicate of each treatment at the beginning of the assay (0 h). After collection, the samples were immediately stored in the dark at a frozen temperature of −20 °C until further analysis. The analytical quantification of the antibiotics was performed according to described by Diogo et al. (2024). The limit of quantification (LOQs) was 1 μg/L for SMX and 0.8 μg/L for TRIM. The method's precision was assessed through the study's repeatability. The compounds studied were not identified in the control samples.

2.5. Fish sacrifice, collection of biological samples, and biochemical biomarkers quantification

After the 28-day exposure period, the organisms were immersed in a rapid ice-cold water bath (≤ 4 °C), and once they exhibited a cessation of opercular movements and swimming ability, they were euthanized by decapitation, following the protocols outlined by Diogo et al., 2023a and Wilson et al., 2009. According to Portuguese animal welfare legislation and the American Veterinary Medical Association's (AVMA) recommendations for animal euthanasia, this process was considered effective, rapid, and not stressful for the fish, and without biochemical disruptions in the organisms (Ministério da Agricultura, 2013; Wilson et al., 2009). After euthanasia, the organisms were measured and weighed. Whole-body tissue was used to assess biomarkers, providing a comprehensive evaluation of the fish's overall physiological status and systemic responses to environmental stressors. For each replicate, two fish bodies (randomly selected) were used for evaluation of antioxidant defense [Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRed) activities, and glutathione (GSH) content], biotransformation [Glutathione S-transferases activity (GSTs)] and lipid peroxidation [Thiobarbituric acid reactive substances (TBARS) levels]. One fish body (randomly selected) was used for quantifications of lactate dehydrogenase (LDH) activity, and another fish body was selected for cellular energy allocation (CEA) assessment, by determining the available energy (Ea): carbohydrates, lipids, and protein total contents and Energy consumed (Ec): electron transport (ETS) activity. One head (randomly selected) was used for the determination of cholinergic neurotransmission (acetylcholinesterase activity – AChE activity). All biological samples (bodies and heads) were immediately stored at −80 °C until the biochemical determinations were quantified following the protocols described in Diogo et al. (2023a) (for SOD, CAT, GPx, GRed, GSTs, LDH, AChE activities, GSH content, and TBARS levels), and Diogo et al. (unpublised data) (for CEA).

2.6. DNA damage determination: comet assay

Gill tissue was selected for DNA damage assessment due to its direct exposure to waterborne contaminants and its higher sensitivity to genotoxic effects, as it is the primary organ for gas exchange, making it particularly vulnerable to genetic damage (e.g., Rodrigues et al., 2016). Gills from one fish per replicate (randomly selected) were collected and immediately processed for genetic damage evaluation, performed according to Rodrigues et al. (2016). A system of six gels per slide was adopted to increase the assay output, based on a model created by Shaposhnikov et al. (2010) and described by Rodrigues et al. (2016). Six microgels of 6 μL were placed on a glass microscope slide, precoated with 1 % normal melting point agarose (NMPA), as two rows of 3 (3 groups of 2 replicates), without coverslips. At the end of the procedure, slides were stored in boxes with light protection, until observation. A Nikon Eclipse Ci fluorescence microscope at 600× magnification, equipped with an excitation filter (540–580 nm) and an emission filter (620–670 nm), was used to assess and quantify the DNA damage. An observation of 100 nucleoids per sample (i.e. replicate) was used to scored/classified into five categories (from 0 to 4), according to the tail and head intensity (Rodrigues et al., 2016). As positive controls, cells of control animals were treated with 50 μM of H_2O_2 for 5 min. The genetic

Table 1
Results of the analytical concentrations of control group (CTL), sulfamethoxazole (SMX), trimethoprim (TRIM), and mixture (MIX) in water samples collected at the beginning of the assay (0 h), and physical and chemical parameters measured during chronic exposure. Established quality criteria for water quality parameters under standard conditions were also presented (OECD, 2000, guideline n° 215).

Treatments		Analytical concentrations (μg/L)	pH	Temp. (°C)	O_2 (%)	Nitrites (mg/L)	Ammonium (mg/L)
(°C)	Nominal (μg/L)						
		Established quality criteria	6.5 to 8.5 ± 0.5	21 to 25 ± 2 °C	> 60 %		
	CTL (SMX = 0.0 TRIM = 0.0)	SMX = 0.0 TRIM = 0.0	7.84 ± 0.07	26 ± 0.3	89.6 ± 0.72	0.283 ± 0.17	0.39 ± 0.25
26	**SMX** (150.0)	150.0	8.01 ± 0.03	26 ± 0.3	94.5 ± 1.25	0.288 ± 0.11	0.31 ± 0.16
	TRIM (30.0)	31.0	7.98 ± 0.04	26 ± 0.2	93.0 ± 1.86	0.274 ± 0.28	0.44 ± 0.32
	MIX (SMX = 150.0 TRIM = 30.0)	SMX = 160.0 TRIM = 32.0	8.01 ± 0.03	26 ± 0.2	92.1 ± 1.70	0.175 ± 0.12	0.20 ± 0.16
	CTL (SMX = 0.0 TRIM = 0.0)	SMX = 0.0 TRIM = 0.0	7.91 ± 0.05	28 ± 0.2	95.0 ± 0.56	0.245 ± 0.13	0.25 ± 0.05
28	**SMX** (150.0)	150.0	8.03 ± 0.05	28 ± 0.4	94.4 ± 0.42	0.471 ± 0.34	0.28 ± 0.15
	TRIM (30.0)	33.5	8.03 ± 0.06	28 ± 0.2	93.0 ± 0.66	0.489 ± 0.34	0.39 ± 0.24
	MIX (SMX = 150.0 TRIM = 30.0)	SMX = 180.0 TRIM = 30.5	8.02 ± 0.02	28 ± 0.4	92.8 ± 1.94	0.267 ± 0.13	0.23 ± 0.19
	CTL (SMX = 0.0 TRIM = 0.0)	SMX = 0.0 TRIM = 0.0	8.02 ± 0.06	32 ± 0.2	93.1 ± 1.70	0.206 ± 0.16	0.40 ± 0.10
32	**SMX** (150.0)	113.9	8.12 ± 0.07	32 ± 0.4	93.1 ± 1.23	0.459 ± 0.38	0.45 ± 0.25
	TRIM (30.0)	43.0	8.08 ± 0.07	32 ± 0.4	92.8 ± 1.57	0.499 ± 0.67	0.26 ± 0.22
	MIX (SMX = 150.0 TRIM = 30.0)	SMX = 150.0 TRIM = 28.5	8.09 ± 0.06	32 ± 0.1	93.6 ± 1.42	0.286 ± 0.16	0.29 ± 0.22

damage index (GDI) was calculated according to Azqueta and Collins (2011), and GDI results were expressed as arbitrary units on a scale of 0–400 per 100 scored nucleoids.

2.7. Data analysis

All biomarkers' data were checked for normality (Shapiro-Wilk test) and homogeneity tests (Levene's test). Before statistical analysis, the CAT, GRed, and GPx activities data were transformed (log(x) + 1 or arcsine) to meet ANOVA assumptions. A two-way ANOVA was performed to assess the combined effects of antibiotics (SMX, TRIM, and MIX) and temperatures (26, 28, and 32 °C). A Dunnett's test was performed to discriminate differences between antibiotic concentrations and the respective control treatment for each temperature. A significance level (α) of 0.05, and SPSS Statistics v29 was used for all the statistical analyses.

2.7.1. Danio rerio: ecotoxicological assessment

After biomarkers quantification, each biomarker's effect percentage was calculated for each antibiotic and temperature treatment relative to the respective control group. This calculation was based on the methodology described by Rodrigues et al. (2022), with adaptations to determine the percentage effect. Ecotoxicity ranges (scores and classes) were established considering the percentage of effects - 10 %, 50 %, and 90 % - according to the methodology of Rodrigues et al. (2022) (Table S2). The results obtained were used to evaluate the toxic effects of each antibiotic treatment at each temperature scenario (Table S2).

2.7.2. Danio rerio: biological health status

A multi-biomarker approach, which combines the responses of different biomarkers, can provide a more precise visualization of consequences caused by various environmental stressors (Piva et al., 2011). According to Li et al. (2019), the biological health status of an organism exposed to different stresses can be classified using the biomarker response index (BRI). This index is based on the degree of alterations in biomarker responses observed in stressed organisms, compared to the normal biological responses (without stress - control group) (Li et al., 2019). For BRI calculation, the relevance factor (W) for each biomarker evaluated in the present study was considered according to their biological relevance and our mechanistic insights (Piva et al., 2011). Thus, the relevance factor 1.0 was used for exposure or effects biomarkers, such as antioxidant defenses (SOD, CAT, GRed, GPx, GSTs activities, and GSH content) and the activities of metabolic enzymes (LDH activity), 1.2 for biomarkers that might preclude adverse effects (TBARS levels), and 1.5 for responses more likely to be prognostic of impairment at higher levels of biological organization (e.g., AChE activity and DNA damage) (Piva et al., 2011). As proposed by Piva et al. (2011), this classification assigns relevance factors to biomarkers depending on their potential to indicate adverse effects, ranging from reversible responses (e.g., antioxidant defenses) to those that predict more severe biological impairment (e.g., DNA damage). After that, the percentage of alterations (AL) caused, compared to the respective control group, was calculated:

$$\mathrm{AL}\ (\%) = \frac{|\mathrm{BR}_{\text{antibiotic treatment}} - \mathrm{BR}_{\mathrm{CTL}}|}{\mathrm{BR}_{\mathrm{CTL}}} \times 100$$

where $\mathrm{BR}_{\text{antibiotic treatment}}$ and $\mathrm{BR}_{\mathrm{CTL}}$ refer to the biomarker (final result of each biomarker) and control group responses, respectively.

Then, all the biomarker responses were distributed into four scores (1 to 4) according to AL (%) obtained (Table S3). Finally, the Biomarker response index (BRI) formula was applied:

$$\mathrm{BRI} = \frac{\sum \mathrm{S_n} \times \mathrm{W_n}}{\sum \mathrm{W_n}}$$

where $\mathrm{S_n}$ and $\mathrm{W_n}$ represent the score and relevance factor of biomarker n, respectively. Furthermore, the *D. rerio* biological health status could be classified as negligible, moderate, major, or severe alterations based on the calculated BRI (Table S3; Hagger et al., 2008).

3. Results

3.1. Water quality

During the chronic exposure, water quality parameters (pH, temperature, conductivity, dissolved oxygen, ammonium, and nitrites) remained within the established quality criteria (OECD, 2000), as shown in Table 1. The analytical concentrations for all treatments are also shown in Table 1. No mortality was observed in the bioassay, meeting the guideline requirements (mortality <10 % in the control group).

3.2. Biomarkers

3.2.1. Antioxidant defense mechanisms and lipid peroxidation

The results of antioxidant and detoxification enzyme activities in *D. rerio* following exposure to environmentally relevant concentrations of SMX, TRIM, and their mixture (MIX) under different temperature conditions (26, 28, and 32 °C) are shown in Fig. 1. Significant interactions were observed between antibiotic concentrations and temperatures for all evaluated parameters (Fig. 1; Table S1).

Taking into account the effects on biomarkers of antioxidant defense, a significant increase in SOD activity was detected following exposure to SMX at 26 and 28 °C, as well as MIX at 28 °C (Fig. 1; Table S1). Conversely, a decrease in SOD activity was observed after exposure to TRIM and MIX at 26 °C, and SMX and MIX at 32 °C (Fig. 1; Table S1). CAT activity showed a significant reduction following exposure to TRIM and MIX at 26 °C, and SMX and TRIM at 28 °C, while a significant increase was noted for MIX at both 28 and 32 °C (Fig. 1; Table S1). Similarly, GRed activity significantly increased after exposure to SMX (at 26 and 28 °C) and MIX (at 28 and 32 °C), and a significant reduction was recorded after exposure to TRIM and MIX at 26 °C (Fig. 1; Table S1). GPx activity increased after SMX at 28 °C, and MIX (at 28 and 32 °C), and was reduced significantly after exposure to all the antibiotic treatments at 26 °C (Fig. 1; Table S1). The activity of GSTs increased after exposure to SMX at 26 and 28 °C, and MIX at 26 and 32 °C, while a significant decrease was observed with TRIM at 26 °C and SMX at 32 °C (Fig. 1; Table S1). GSH content followed a pattern similar to GRed activity, except for TRIM at 32 °C, which significantly increased GSH levels without affecting GRed activity. TBARS levels significantly increased after SMX exposure at 26 and 28 °C, and MIX at 28 and 32 °C (Fig. 1; Table S1).

3.2.2. Cholinergic neurotransmission

Significant interactions were observed between antibiotic concentrations and temperature conditions for AChE activity (Fig. 1; Table S1). AChE activity significantly decreased after chronic exposure to SMX, TRIM, and MIX across different temperature scenarios, except for TRIM at 32 °C, which caused a significant increase in AChE activity (Fig. 1 and Table S1).

3.2.3. Energetic metabolism

Significant interactions were observed between antibiotic concentrations and temperature conditions for all pathways of obtaining energy (LDH activity and energy reserve contents; Fig. 1). A significant decrease in LDH activity was observed following exposure to all antibiotic treatments at 26 °C and SMX at 28 °C. In contrast, a significant increase in LDH activity was noted for MIX at 28 °C and SMX at 32 °C. A similar response was also detected in carbohydrates content and Ea after exposure to SMX and MIX at 28 °C (significant increase), and SMX at 32 °C, while a significant decrease occurred in SMX and TRIM at 26 °C, and MIX at 32 °C. A significant increase occurred in lipid content only after exposure to MIX at 32 °C. Regarding protein content, a significant decrease in all the antibiotic treatments at 26 °C and TRIM at 32 °C was

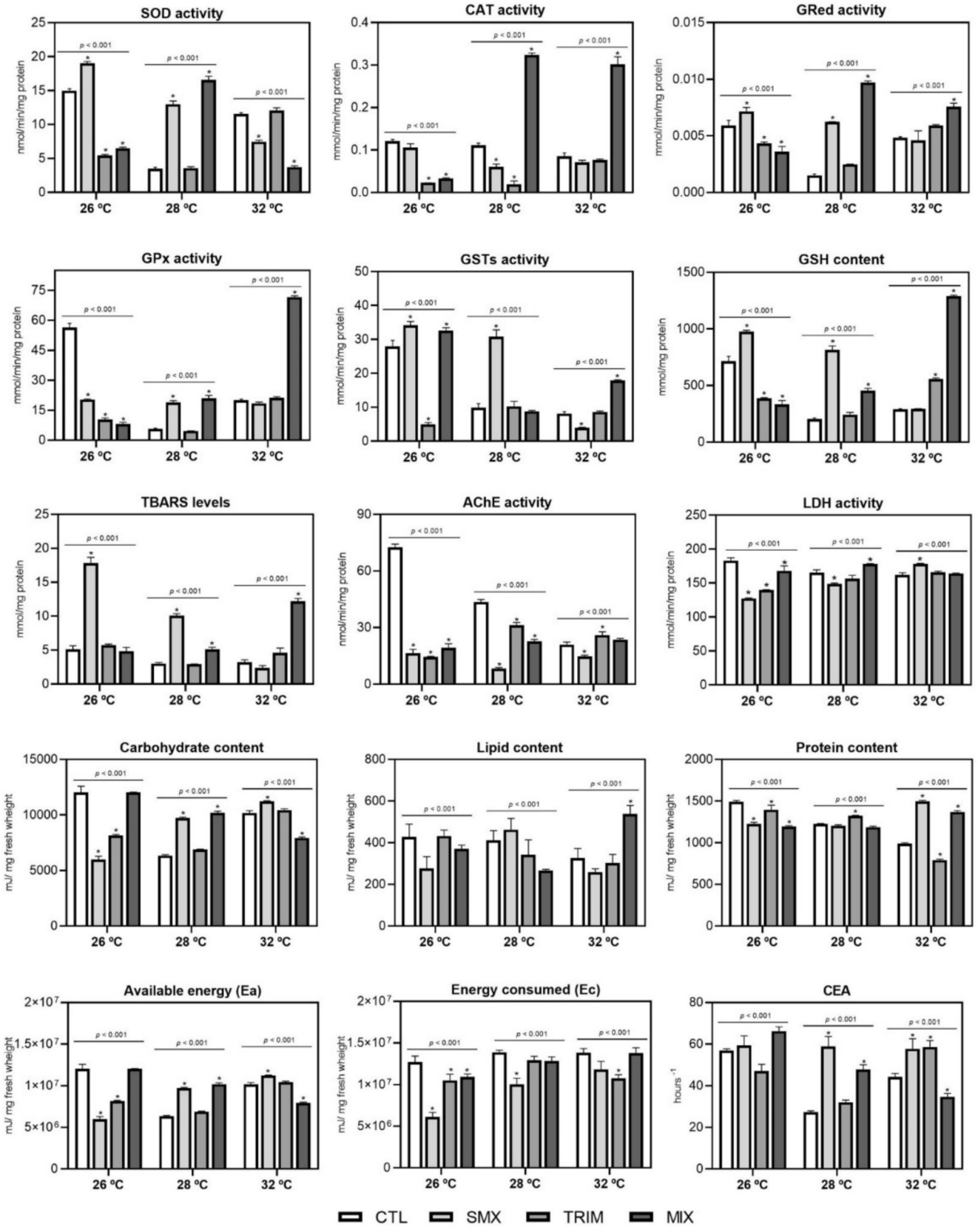


Fig. 1. Results of biochemical biomarkers of *Danio rerio* after chronic exposure (28 days) to sulfamethoxazole (150 μg SMX/L), trimethoprim (30 μg TRIM/L), and mixture (MIX = 150 μg SMX/L + 30 μg TRIM/L) in different temperature scenarios (26, 28, and 32 °C). Data are expressed as mean (n = 3) ± standard error bars. Significant effects (*p* level) of antibiotics for each temperature are shown. Asterisks (*) discriminate significant differences between the control group and antibiotic treatments in each temperature (Dunnett's test; $p < 0.05$).

observed, while the opposite occurred after exposure to TRIM at 28 °C and SMX and MIX at 32 °C. Ec decreased significantly in almost treatments, namely at 26 °C, SMX at 28 °C, and TRIM at 32 °C. Additionally, CEA levels significantly increased after exposure to SMX and MIX at 28 °C, and SMX and TRIM at 32 °C. Only MIX at 32 °C caused a significant decrease in CEA (Fig. 1 and Table S1).

3.2.4. DNA damage determination

Significant interactions were observed between antibiotic concentrations and temperatures for the genetic damage index (Fig. 2). Overall, for all temperature conditions, organisms exposed to SMX and TRIM exhibited a higher percentage of DNA damage in classes 1 and 2 (Fig. 2A and 2B). In contrast, organisms exposed to MIX showed a higher incidence of damage in more severe classes 3 and 4 (Fig. 2B), regardless of the temperature. The GDI revealed a significant increase after exposure to all the antibiotic treatments across all temperature scenarios (Fig. 2C and Table S1).

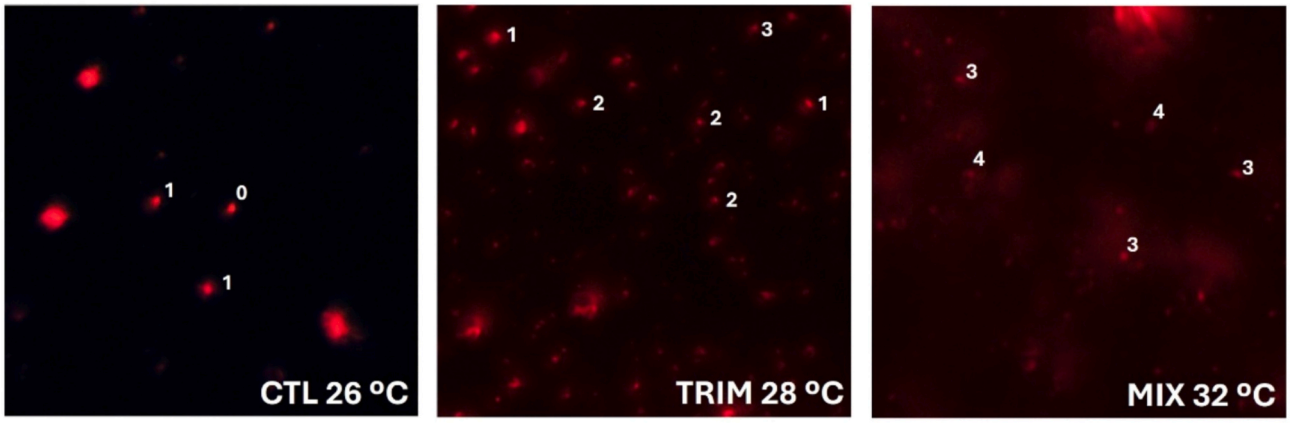
3.3. Antibiotics ecotoxicological assessment and biological health status

Table 2 shows the percentage of effect (2A.1) of all biomarkers evaluated, and the final toxicity classification (2A.2) obtained in *D. rerio* after exposure to environmentally relevant concentrations of SMX, TRIM, and their MIX, under different temperature conditions (26, 28, and 32 °C; Table S2). The BRI values (2B) obtained (Table 2 and Table S2), and the respective biological health status were also presented. Regarding the final toxicity evaluation (Table 2A, 2B, and Table S2) at 26 °C, all antibiotic treatments (SMX, TRIM, and MIX) were classified as marginally toxic, and induced major alterations (e.g., oxidative stress and neurotoxicity) in *D. rerio* ($2.51 \leq BRI \leq 2.75$; Table S3). At 28 °C, TRIM was considered slightly toxic, and caused negligible alterations (e.g., alterations in antioxidant defense) to the biological health status of zebrafish ($3.01 \leq BRI \leq 4.00$; Table S3). On the other hand, SMX and MIX at this temperature were moderately toxic, and provoked severe alterations (e.g., DNA damage) in *D. rerio* health status (BRI ranging from 1.00 to 2.50; Table S3). At 32 °C, SMX and TRIM were the treatments with lower effects, classified as slightly toxic, and caused negligible alterations to zebrafish biological health ($3.01 \leq BRI \leq 4.00$; Table S3). In contrast, MIX at 32 °C exhibited moderate toxicity, and induced severe alterations in *D. rerio* health status (BRI ranging from 1.00 to 2.50; Table S3).

4. Discussion

Temperature is a known stressor that can modulate the toxicity of compounds (e.g., pharmaceuticals and pesticides) and alter the organism's sensitivity (Wiles et al., 2020), by affecting physiological processes, enzymatic activity, membrane permeability, and the uptake, biotransformation, and elimination of contaminants (Kazmi et al., 2022; Macek et al., 1969; Zhou et al., 2014). The results of the present study suggest that temperature influences the physiological responses of *D. rerio* to the antibiotics SMX, TRIM, and MIX, affecting antioxidant

A)



B)

Treatments		Damage classes				
		0	1	2	3	4
26 °C	CTL	59.7 ± 3.8	40.3 ± 3.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	SMX	6.00 ± 0.0	78.0 ± 0.8	16.0 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
	TRIM	13.7 ± 0.0	61.0 ± 0.6	25.3 ± 0.5	0.0 ± 0.0	0.0 ± 0.0
	MIX	0.0 ± 0.0	6.00 ± 0.1	47.3 ± 0.9	41.0 ± 1.2	5.70 ± 0.2
28 °C	CTL	25.0 ± 2.1	75.0 ± 2.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	SMX	2.70 ± 0.0	60.7 ± 0.6	36.7 ± 0.7	0.0 ± 0.0	0.0 ± 0.0
	TRIM	0.0 ± 0.0	11.3 ± 0.1	55.3 ± 1.1	33.3 ± 1.0	0.0 ± 0.0
	MIX	0.0 ± 0.0	1.0 ± 0.0	16.3 ± 0.3	64.0 ± 1.9	0.0 ± 0.0
32 °C	CTL	36.0 ± 4.6	64.0 ± 4.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	SMX	3.30 ± 0.0	65.3 ± 0.7	31.0 ± 0.6	0.30 ± 0.0	0.0 ± 0.0
	TRIM	0.0 ± 0.0	4.30 ± 5.5	64.0 ± 1.3	31.7 ± 1.0	0.0 ± 0.0
	MIX	0.0 ± 0.0	0.0 ± 0.0	21.3 ± 0.4	46.0 ± 1.4	32.7 ± 1.3

C)

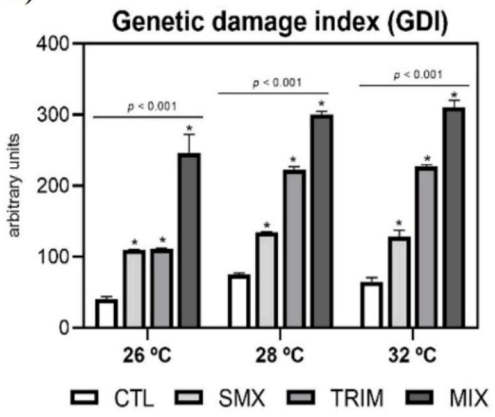


Fig. 2. A) Representative photographs of comet assay damage classes observed in gills of *Danio rerio* after chronic exposure (28 days) to sulfamethoxazole (150 μg SMX/L), trimethoprim (30 μg TRIM/L), and mixture (MIX = 150 μg SMX/L + 30 μg TRIM/L) in different temperature scenarios (26, 28, and 32 °C); B) Results of percentage of damage classes; C) Genetic Damage Index (GDI, expressed as arbitrary units). Data are expressed as mean ($n = 3$) ± standard error bars. Significant effects (p level) of antibiotics for each temperature are shown. Asterisks (*) discriminate significant differences between the control group and antibiotic treatments in each temperature (Dunnett's test; $p < 0.05$).

Table 2
Results of A1) Percentage of effects of each biomarker evaluated in *D. rerio* after chronic exposure to environmentally relevant concentrations of sulfamethoxazole (150 μg SMX/L), trimethoprim (30 μg TRIM/L), and mixture (MIX = 150 μg SMX/L + 30 μg TRIM/L) in different temperature scenarios (26, 28 and 32 °C). A2) Ecotoxicity scores (1 to 5) and the final ecotoxicological classification [slightly toxic (ST - green), marginally toxic (MGT - yellow), and moderately toxic (MT - orange)] obtained for each antibiotic treatment (*see* Table S2). B) Biomarker response index (BRI) and the respective classification of biological health status were also presented. BRI values: 1.00 ≤ BRI ≤ 2.50 - severe alterations (red); 2.51 ≤ BRI ≤ 2.75 - major alterations (orange); 2.76 ≤ BRI ≤ 3.00 - moderate alterations (yellow); 3.01 ≤ BRI ≤ 4.00 - negligible alterations (green) (*see* Table S3).

Biochemical biomarkers	A1) Effects (%)								
	26 °C (standard)			28 °C			32 °C		
	SMX	TRIM	MIX	SMX	TRIM	MIX	SMX	TRIM	MIX
SOD activity	27.0	-63.8	-56.8	271.8	2.19	374.3	-35.5	4.30	-67.8
CAT activity	-12.0	-81.2	-72.5	-46.0	-82.5	190.8	-16.5	-10.6	254.1
GRed activity	20.8	-26.8	-39.4	311.8	63.9	541.0	-5.15	22.3	56.3
GPx activity	-63.9	-81.6	-85.6	233.6	-18.4	272.8	-7.64	6.16	258.3
GSH content	36.4	-45.7	-53.2	299.7	18.5	122.3	0.77	91.0	342.6
GSTs activity	22.3	-82.2	17.0	212.4	3.95	-10.8	-51.4	5.52	120.3
TBARS levels	249.5	12.6	-4.97	233.1	-2.73	68.3	-26.2	42.5	277.0
AChE activity	-77.2	-80.1	-73.3	-81.3	-28.4	-47.8	-29.8	24.2	12.8
LDH activity	-30.4	-23.5	-8.22	-10.0	-5.47	7.86	9.89	2.25	1.09
CEA	42.3	-17.4	16.4	115.2	17.2	74.8	30.3	32.3	-21.6
GDI	172	177	511	78.7	196	300	96.4	248	377
A2) Final toxicity	**3 MGT**	**3 MGT**	**3 MGT**	**4 MT**	**2 ST**	**4 MT**	**2 ST**	**2 ST**	**4 MT**
B) Biomarker Response Index									
BRI	**2.60**	**2.61**	**2.65**	**1.66**	**3.18**	**1.92**	**3.20**	**3.08**	**2.02**
Biological Health Status	**Major**	**Major**	**Major**	**Severe**	**Negligible**	**Severe**	**Negligible**	**Negligible**	**Severe**

defense, cholinergic neurotransmission, and energy pathways (Fig. 1). These findings suggest that environmental temperature plays a critical role in modulating both the efficacy and toxicity of the antibiotics to aquatic organisms.

4.1. Antioxidant defense mechanisms and lipid peroxidation

Under IPCC-predicted warming scenarios (28 and 32 °C), distinct antioxidant and lipid peroxidation responses were observed in *D. rerio* exposed to antibiotics (Fig. 1). While individual antibiotics (SMX and TRIM) showed variable effects with temperature, their combination (MIX) consistently led to heightened toxicity, with increased oxidative stress and overwhelmed antioxidant defenses at higher temperatures. These findings suggest that rising temperatures may exacerbate the toxic effects of antibiotic mixtures, posing greater risks to non-target species, like *D. rerio* (Jesus et al., 2018; Mehta, 2017). On a broader scale, the combined stress of warming and pharmaceutical pollution could further destabilize aquatic ecosystems by impairing organism health, disrupting trophic interactions, and weakening overall ecosystem resilience (Mehta, 2017). SMX exposure induced oxidative stress in *D. rerio*, with varying degrees of intensity depending on temperature (Fig. 1). These findings suggest that SMX triggers the production of reactive oxygen species (ROS) and compromises the antioxidant capacity of *D. rerio*, as previously reported by other authors (e.g., Huo et al., 2023; Yan et al., 2016). Yan et al. (2016) studied the effect of 200 μg SMX/L at 28 °C in zebrafish, observing severe oxidative stress (induction of metabolic enzyme activity) after long-term exposure (150 days). Similarly, Iftikhar and Hashmi (2021) found that 28 days of SMX exposure (25, 50, 100, and 200 μg/L), at temperatures between 26 and 28 °C, led to ROS production in a dose and time-dependent manner in *Cyprinus carpio*. Li et al. (2012) also reported a significant increase in GSTs activity in *Carassius auratus* exposed to SMX (> 16 μg/L at 18 °C). Ramesh et al. (2018) observed that 1000 μg/L of sulfamethazine (an antibiotic from the same group as SMX) at 27 °C disrupted the antioxidant defense system in *Cirrhinus mrigala*, altering the activities of SOD, CAT, and GPx.

TRIM exposure also revealed that temperature modulates the organism's physiological response, with the antibiotic causing more extensive suppression of antioxidant defenses at standard temperatures for *D. rerio* (26 °C) but having a reduced impact at higher temperatures (28 and 32 °C; Fig. 1). This temperature-dependent response could be attributed to differences in metabolic activity, enzyme kinetics, or compensatory stress responses across the temperature gradient. Supporting these findings, Diogo et al. (2024) found that *D. rerio* exposed to TRIM at 28 °C (≤ 400 mg/L) showed decreased CAT activity without increased TBARS levels. Similarly, Fernandez et al. (2022) reported that 21 days of exposure to 10 μg TRIM/L at 19 °C increased CAT and GRed activities in *Sparus aurata*, while TBARS levels remained unaffected, leading to oxidative stress.

Regarding MIX treatment, at 26 °C, antioxidant defenses were notably suppressed, while at 28 and 32 °C, *D. rerio* adopts a more active antioxidant response, likely as an adaptation to increased metabolic stress (Fig. 1). While research on SMX and TRIM mixture in *D. rerio* is

limited, several studies have shown that antibiotic mixtures can disrupt key physiological pathways in various aquatic species (e.g., Carlsson et al., 2013; Iftikhar et al., 2023; Oliveira et al., 2013; Tokanová et al., 2021; Yildiz and Altunay, 2011). For example, Yang et al. (2019) studied the combined effect of three pharmaceuticals (ofloxacin, SMX, and ibuprofen) on *Carassius auratus,* and they found that the antioxidant response varies with the concentration of each compound in the mixture. Similarly, Yan et al. (2016) reported that the combination of SMX and norfloxacin in *D. rerio* activated antioxidant and metabolic enzyme activities to scavenge ROS at 28 °C. Moreover, Madureira et al. (2012) observed liver cytohistological changes in zebrafish exposed to TRIM and other pharmaceuticals (e.g., SMX, carbamazepine, fenofibric acid, and propranolol), highlighting the potential for broader ecological impacts in aquatic environments.

4.2. Cholinergic neurotransmission

The tested antibiotics showed temperature-dependent effects on AChE activity. Notably, basal AChE levels varied significantly across temperature controls (Fig. 1), indicating that temperature alone can influence neurocholinergic function. At 28 °C, all antibiotics significantly inhibited AChE activity; however, at 32 °C, responses diverged (SMX still reduced AChE activity, TRIM induced a significant increase, and MIX had no significant effect). This suggests that, under elevated temperatures, the antibiotic mixture may stabilize AChE activity, potentially mitigating neurotoxic compared to individual antibiotics. Such stabilization may reflect a compensatory response to combined thermal and chemical stress. Nonetheless, the divergent responses at 32 °C highlight the complexity of predicting neurotoxicity under warming scenarios. These findings underscore the risk of altered neurotransmission in aquatic species, potentially affecting vital behaviors like locomotion, feeding, escape, and reproduction, and ultimately threatening individual survival and ecosystem stability (Muñoz-Peñuela et al., 2022).

Multiple studies have shown that different antibiotics (e.g., norfloxacin, erythromycin, sulfadiazine, sulfisoxazole) affected AChE activity in aquatic organisms, at standard temperatures, disrupting normal neurological functions and potentially leading to adverse effects (such as behavioral disturbances, development, and defense mechanisms) (Huo et al., 2023; Liu et al., 2014; Rodrigues et al., 2019; Yan et al., 2016). In the present study, AChE activity decreased significantly after exposure to all the antibiotics, regardless of temperature conditions (except TRIM and MIX at 32 °C), indicating a neurotoxic response (Fig. 1). According to US EPA (2000), a $\geq$ 20 % reduction in AChE activity is considered both biologically and statistically relevant, signaling physiological dysfunction. In this work, inhibition consistently exceeded that threshold, particularly at 26 and 28 °C (Table 2), confirming a marked biological effect. These results align with prior findings in *D. rerio* embryos exposed to SMX at 28 °C (Liu et al., 2020) and with studies in *C. auratus*, where mixtures of SMX and norfloxacin caused 35 % AChE inhibition at 18 °C (Liu et al., 2014). Similarly, Li et al. (2012) observed an inhibition of AChE activity in *C. auratus* after 7 days of exposure to $\geq$400 μg SMX/L at lower temperatures (16–18 °C). In line with this, Diogo et al. (2025) reported a similar temperature-mediated response in *Daphnia magna* exposed to TRIM (30 μg/L) at 20 °C, and after exposure to SMX (> 0.156 μg/L at 28 °C) in zebrafish embryos (Diogo et al., 2024). Both SMX and TRIM are antifolates that disrupt folate biosynthesis, impairing cell proliferation (Masters et al., 2003). Huo et al. (2023) reported that antibiotics with this mechanism of action (e.g., sulfonamides group) can affect neurotransmitters and cause endocrine disruptions by interfering with folate and carbonic anhydrase pathways. Since folate is essential for the methionine cycle and metabolically linked to choline (a precursor of acetylcholine), its inhibition may limit acetylcholine synthesis, indirectly altering AChE activity (Crivello et al., 2010; Lee et al., 2012). This connection reinforces the hypothesis that antifolate antibiotics can disrupt neurocholinergic function through indirect metabolic interference.

4.3. Energy metabolism

Under the warming scenarios predicted by IPCC (28 and 32 °C), both individual and combined antibiotic exposure significantly disrupted the energy metabolism of *D. rerio*, potentially reducing organism fitness and altering ecological interactions. These metabolic disruptions, driven by increased energy demands and compromised energy balance, may impair critical functions such as growth, reproduction, and survival (Bethke et al., 2023; Jesus et al., 2018). At a broader scale, such combined stressors threaten ecosystem stability by affecting higher levels of biological organization, posing risks to population dynamics and biodiversity (Smolders et al., 2009). Temperature is known to influence metabolic pathways related to energy production (Lemieux and Blier, 2022; Sokolova and Lannig, 2008). In this study, antibiotic exposure at different temperatures altered energy reserves, particularly carbohydrates and proteins, with the most notable changes observed in these fractions (Fig. 1). Additionally, the results indicate that the organism needs more energy to cope with the elevated temperature when present to other stress factors, such as antibiotics (Fig. 1). Despite specific studies on the CEA of zebrafish juveniles exposed to antibiotics at varying temperatures being lacking, Diogo et al. (unpublished data) reported significant stress in zebrafish embryos exposed to SMX (156, 313, and 625 μg/L, at 26 °C). This stress (SMX at 26 °C) was evidenced by a marked decrease in Ea and CEA, demonstrating a reallocation of energy to detoxification processes (e.g., cellular repair and maintenance). The same authors reported that TRIM (< 400 mg/L, at 26 °C) did not affect Ea and Ec, but also caused a significant decrease in CEA of zebrafish embryos (Diogo et al., unpublished data). Supporting these findings, Yildiz and Altunay (2011) showed that SMX–TRIM mixtures triggered metabolic stress in fish (*Dicentrarchus labrax* and *Sparus aurata)*, increasing glucose via glycogenolysis and gluconeogenesis. Similarly, Verslycke and Janssen (2002) found that temperature significantly impacted energy reserves in *Neomysis integer*, more so than other environmental factors (e.g, salinity or dissolved oxygen). These results reinforce the idea that temperature is a key modulator of energy metabolism, especially under chemical stress.

Previous studies have shown that LDH activity, a key enzyme in anaerobic energy production (Dar et al., 2025), is influenced by temperature (Farhana and Lappin, 2024; Zakhartsev et al., 2004). Exposure to antibiotics also influenced LDH activity, and to all antibiotics tested at 26 °C, and to SMX at 28 °C, LDH activity significantly decreased. However, MIX and SMX at higher temperatures led to activation of anaerobic metabolism to meet elevated energy demands and maintain vital functions (Fig. 1). A similar pattern was reported by Schnurr et al. (2013), who observed increased LDH activity in zebrafish exposed solely to higher temperatures (32 °C), indicating temperature-driven metabolic adjustments. Additionally, Matozzo et al. (2015) reported an increase in LDH activity in clams *Ruditapes philippinarum* exposed to TRIM (0.3, 0.6, and 0.9 ng/L; at 17 °C), indicating possible immune suppression and cell damage. These results suggest that both antibiotics and elevated temperatures can disrupt energy metabolism, leading to increased reliance on anaerobic pathways.

4.4. DNA damage

GDI values corroborated a significant rise in genotoxicity across all thermal scenarios and antibiotics exposure (Fig. 2). These findings highlight that, under warming conditions, the combination of SMX and TRIM poses a greater genotoxic risk than the individual compounds, potentially impairing vital cellular functions, development, and immune competence in *D. rerio*. Such effects may compromise population stability and trigger broader ecological consequences, including altered ecosystem dynamics, food web disruptions, and biodiversity loss, underscoring the urgent need for stricter regulation of pharmaceutical

contaminants in a changing climate. The GDI results show that exposure to SMX, TRIM, and MIX caused DNA damage in the gills of *D. rerio*, with damage severity at higher temperatures (Fig. 2B, 2C; and Table S1). This DNA damage is likely linked to oxidative stress caused by the failure of antioxidant defenses under thermal stress (Fig. 1), leading to ROS overproduction and subsequent damage to cellular macromolecules, including DNA (Dar et al., 2024; Nunes et al., 2019). Supporting these findings, Hassan (2017) reported that both suboptimal cold (14 °C) and heat stress (36 °C) in *Oreochromis niloticus* altered the expression of genes linked to oxidative stress indicators (e.g., metallothioneins, glutathione transferases), immune function, and heat shock response (HSP70). Similarly, Buschini et al. (2003) found that temperature influenced DNA damage levels in *Dreissena polymorpha*, modulating cellular sensitivity to pollutants even under in vitro conditions. Other studies revealed that DNA modifications in fish from unpolluted waters were similar to those observed in fish from polluted environments, but under different temperature conditions, suggesting that natural factors, such as temperature, significantly contribute to DNA damage (Kurelec et al., 1989).

The potential for cumulative effects from natural (e.g., temperature, pH) and anthropogenic sources (e.g., antibiotics, pesticides) remains a concern for fish populations, as most studies isolate single factors rather than examining their combined impacts. Antibiotics have been shown to induce significant DNA damage (Papis et al., 2011; Ramesh et al., 2018; Sharma et al., 2021), raising concerns about their ecological consequences, especially regarding organismal health, reproduction, and survival. The genotoxicity observed in the present study appears closely linked to the mechanism of action of SMX and TRIM, which function as folic acid antagonists by inhibiting dihydrofolate reductase (Masters et al., 2003). This inhibition disrupts the synthesis of tetrahydrofolic acid, a precursor essential for thymidine and, consequently, DNA synthesis (Masters et al., 2003; Sangurdekar et al., 2011). Such interference in nucleotide production compromises DNA replication and repair, potentially leading to mutations, apoptosis, or carcinogenesis (Mason and Levesque, 1996). Although studies specifically addressing SMX-induced DNA damage in fish are limited, several authors have demonstrated that SMX presence in aquatic environments can induce oxidative stress, a known driver of DNA damage (Papis et al., 2011; Polianciuc et al., 2020; Rocco et al., 2012). Similarly, TRIM has been shown to induce DNA strand breaks in rainbow trout cells, particularly at higher concentrations (100 mg/L) (Papis et al., 2011), and caused significant DNA damage in *Mytilus edulis* at 200 mg/L (Lacaze et al., 2015). Nevertheless, Liu et al. (2014) demonstrated that a mixture of norfloxacin and SMX (5 mg/L + 25 mg/L) also induced DNA damage in *Carassius auratus* after seven days of exposure at 19 °C, reinforcing the concern over mixture effects.

5. Ecotoxicity classes and biological health status

The ecotoxicity classification and biomarker response index provide a comprehensive view of biological responses, enabling a detailed evaluation of the potential risks associated with these co-exposures (Li et al., 2019). Our findings indicate that environmentally relevant concentrations of SMX, TRIM, and MIX significantly affect the health of juvenile *D. rerio*, regardless of temperature. At standard temperature, the antibiotics tested, individually and in the mixture, were marginally toxic and caused major alterations in the biological health status of this species (Table 2). Evaluating the individual effects of SMX and TRIM under higher temperature conditions, antibiotics displayed different toxic effects depending on the degree of warming. These findings suggest that, in more extreme warming scenarios (e.g., a 6 °C increase above standard temperatures), neither SMX nor TRIM significantly intensifies their toxic effects, posing a relatively low impact on the species' overall health. In contrast, the MIX at 28 °C and 32 °C was moderately toxic and induced severe alterations in *D. rerio* juveniles (Table 2). These results demonstrate that, under increased temperatures, the combined exposure to SMX and TRIM was more harmful, significantly disrupting the physiological and biochemical functions of the fish. The increased toxicity of the MIX compromises homeostasis, causing irreversible cellular damage and further impairing the organism's health. This, in turn, affects its ability to adapt, grow, and survive.

This study offers a significant contribution by addressing the interaction between antibiotics and global warming, focusing on environmentally relevant concentrations and their consequences for the health of *D. rerio*. These findings emphasized the critical issue of chemical mixtures and the escalating impact of rising temperatures on aquatic ecosystems. They underscore the urgent need to understand how the combined effects of contaminants and climate change influence the health and survival of aquatic organisms. By using a multiple-biomarker approach, the results provide new insights into how rising temperatures can amplify the toxic effects of contaminants, highlighting the urgent need to study the synergy between environmental stressors to protect aquatic ecosystems. The increased toxicity observed at elevated temperatures underscores the amplified risks to aquatic organisms due to the combined impacts of global warming and contaminant exposure. As global temperatures continue to rise, the vulnerability of aquatic environments to contaminant mixtures is likely to grow, emphasizing the need for in-depth studies to guide practical regulatory actions and conservation efforts. The inclusion of the BRI and ecotoxicity classification enables a more integrated and ecologically relevant assessment of contaminant effects by translating complex biological responses into environmental risk levels. These approaches are essential for understanding the combined impacts of pollutants and environmental stressors, such as temperature, under realistic ecological scenarios. Moreover, these approaches facilitate comparison across different studies and species by providing standardized metrics, enhancing the consistency and applicability of ecotoxicological data under realistic environmental scenarios. The toxic synergy between rising temperatures and antibiotic pollution severely threatens aquatic life's resilience and the stability of ecosystem functions, urging immediate attention and action to mitigate these impacts.

CRediT authorship contribution statement

Bárbara S. Diogo: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Sara Rodrigues:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Bent Speksnijder:** Methodology. **Oksana Golovko:** Writing – review & editing, Methodology. **Sara C. Antunes:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization.

Consent to participate

Not applicable.

Consent for publication

The paper is submitted with the mutual consent of the authors for publication.

Ethical approval

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2025.110240.

Data availability

All data are present in the manuscript

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