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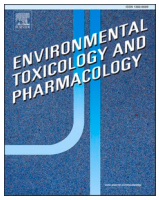

Dual environmental stressors: How pH modulates antibiotic toxicity in *Danio rerio*?

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ABSTRACT

Aquatic ecosystems are increasingly subjected to environmental stressors, including pH fluctuations, and antibiotic contamination, which can disrupt essential biological functions such as metabolism, respiration, and reproduction. The interaction between these stressors presents significant ecological risks, as pH affects the toxicity, pharmacodynamics/kinetics of antibiotics by altering their ionization state and membrane permeability. This study assessed the toxicity of environmentally relevant concentrations of sulfamethoxazole (150 μg SMX/L), trimethoprim (30 μg TRIM/L), and their mixture (MIX: 150 μg SMX/L + 30 μg TRIM/L) under different pH conditions (6.5, 7.5, and 9.0) on *Danio rerio* juveniles. A multi-biomarker approach was used to assess *D. rerio* biological health status, including oxidative stress responses, lipid peroxidation, cholinergic neurotransmission, energetic metabolism, and DNA damage. Results revealed that SMX was marginally toxic across all pH scenarios, but caused more severe effects such as oxidative stress, lipid peroxidation, and DNA damage, under acidic pH. In contrast, TRIM toxicity increased at neutral and alkaline pH, causing severe alterations in antioxidant defenses and cellular integrity. The MIX treatment exhibited marginal toxicity at acidic and alkaline pH but was moderately toxic at neutral pH, leading to oxidative stress, lipid peroxidation, and DNA damage. These physiological and metabolic disruptions highlight how antibiotic mixtures, under varying pH conditions, can impair critical biological functions in aquatic organisms. These findings emphasize the urgent need for integrated research addressing multiple environmental stressors, particularly chemical contamination and climate change-driven abiotic factors. Ignoring these threats could lead to irreversible damage to aquatic ecosystems and biodiversity.

1. Introduction

"Anthropocene" describes the era characterized by profound environmental changes caused by human activity, especially the increase of burning fossil fuels and CO_2 emissions over the last two centuries, which have impacted the global climate (Giorgi, 2024; Thomas et al., 2022; Vargas et al., 2017). Excess carbon is absorbed by the oceans, leading to acidification through increased hydrogen ion concentrations and decreased pH values (Birchenough et al., 2017; Vargas et al., 2017). Since the Industrial Revolution, about 48 % of the CO_2 produced by human activities has been absorbed by the oceans (Sabine et al., 2004), leading to a pH drop from 8.2 to 8.1, and projected to a further decrease by 0.3–0.4 units by the end of the century (IPCC, 2023; Thomas et al., 2022).

Ocean acidification has been widely studied (Birchenough et al., 2017), due to its harmful effect on marine organisms, also causing habitat loss and population declines (IPCC, 2023). Although the acidification mechanism may be similar in oceans and freshwater, some factors specific (e.g., buffering capacity, greater seasonal pH variability) in freshwater systems make it difficult to understand acidification caused by the atmospheric CO_2 increase (Thomas et al., 2022). The pH of freshwater systems tends to become more acidic, and a change of 0.3–0.5 is expected in large freshwater bodies, occurring more quickly than in the ocean (Weiss et al., 2018). However, some studies report recent increases in freshwater pH (alkalinization) in reservoirs and rivers in Portugal and the U.S., with shifts of about 1 unit (pH > 8) (e.g.,

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INAG, 2011a, 2011b, 2011c; Kaushal et al., 2018, 2013; Pinto et al., 2025). Considering these changes, understanding the biological effects of pH fluctuations in freshwater ecosystems is essential.

pH fluctuations in freshwater ecosystems can disrupt vital physiological functions across species, from invertebrates to fish, by altering nutrient availability, cell membrane properties, and chemical toxicity (Alsop and Wilson, 2019; Nakamura et al., 2008; Valenti et al., 2009). Even slight pH changes can alter the chemical properties of compounds, such as antibiotics, by modifying their solubility in the medium, transport, bioavailability, and toxicity, which in turn may impact community composition (Almeida et al., 2022; Bethke et al., 2023; Suter et al., 2023). The co-occurrence of pH fluctuations and antibiotic contamination presents a particularly concerning scenario, as both factors may interact, amplifying the potential for environmental harm. The pharmacodynamics or pharmacokinetics of antibiotics can vary with pH, as the degree of ionization of these compounds and their membrane permeability depend directly on the environmental conditions (Zhang et al., 2023). Furthermore, deviations in pH levels from an organism's optimal range can heighten its susceptibility to the toxic effects of these substances by disrupting essential physiological processes, such as enzyme activity, membrane integrity, and ion balance, thereby potentially amplifying ecotoxicological damage (Bethke et al., 2023).

Given the significant influence of pH on the pharmacodynamics or pharmacokinetics of pharmaceuticals, it is crucial to examine the environmental impact of antibiotics under different pH values scenarios (due to climatic changes), frequently detected in different aquatic matrices. Among these, sulfamethoxazole (SMX) and trimethoprim (TRIM) have been found in various environmental compartments with concentrations ranging from ng to μg/L (e.g., freshwater, seawater, and groundwater) (Carvalho and Santos, 2016; Duan et al., 2022). Both antibiotics are also listed as priority substances by the Watch List under the Water Framework Directive, underscoring the need for further investigation into their toxicity (Cortes et al., 2020, 2022). SMX and TRIM are among the most frequently detected antibiotics in aquatic environments, particularly in surface waters near urban or agricultural areas, where concentrations can reach up to 150 μg/L for SMX and 30 μg/L for TRIM (Kairigo et al., 2020; Khan et al., 2013), and their common co-occurrence can often be a part of fixed-dose pharmaceutical combinations. Clinically, these antibiotics are commonly administered together as a synergistic combination therapy due to their complementary modes of action on bacterial DNA synthesis (SMX inhibits dihydropteroate synthase, while TRIM targets dihydrofolate reductase) (Daeseleire et al., 2017; Masters et al., 2003). Despite their known synergy in medical contexts, the ecological consequences of their co-occurrence remain poorly understood. Although previous studies have reported various toxic effects of SMX and TRIM (e.g., neurotoxicity and reproductive impairment) in aquatic organisms (e.g., *Daphnia magna* and *Danio rerio*), these often focus on isolated exposures under standard laboratory conditions (Dionísio et al., 2020; Park and Choi, 2008). However, little is known about how these compounds interact under varying environmental parameters, such as pH. This study addresses this knowledge gap by evaluating the combined and individual effects of SMX and TRIM across a realistic pH gradient using *D. rerio*, a well-established ecotoxicological model for freshwater ecosystems (OECD, 2000). By integrating environmentally relevant concentrations with ecologically meaningful pH scenarios, this study contributes to a more accurate assessment of ecological risks in the context of ongoing climate-related pH changes. Therefore, this study aims to evaluate the toxicity of environmentally relevant concentrations of SMX, TRIM, and their mixture (MIX) under different pH conditions (6.5, 7.5, and 9.0) on the fish species *D. rerio*. To achieve this goal, a multi-biomarker approach was applied to assess the organism's biological health. The study provides novel insights into the intricate interactions between antibiotics and pH variations, highlighting the pH-dependent toxicological responses and potential synergistic effects. This will enhance our understanding of complex environmental dynamics that affect water quality, biodiversity, and the resilience of aquatic ecosystems to antibiotics.

2. Material and methods

2.1. Study organism - Danio rerio

Danio rerio (zebrafish) is a freshwater species commonly used as a model organism for evaluating the impact of contaminants on aquatic ecosystems (OECD, 2000). Juvenile *D. rerio* used in the experiment were sourced from a laboratory broodstock and reared under standard conditions at CIIMAR's certified laboratory facilities, the Interdisciplinary Centre of Marine and Environmental Research in Matosinhos, Portugal. The acclimation period (three weeks) was carried out in 60 L tanks with continuous aeration, and dechlorinated tap water, under controlled conditions of photoperiod (16 h light/8 h dark), temperature (26 ± 1 °C), and pH (7.5 ± 0.5). Several water parameters were monitored, temperature, conductivity, dissolved oxygen, ammonium, and nitrite levels, every two days to ensure water quality (criteria established present in Table 1). The organisms were fed *ad libitum* with commercial zebrafish food (Zebrafeed 400–600 μm by Sparos) and were deemed suitable and healthy for the experiments, as no signs of disease or mortality were observed (at least 15 days before the assays). All the procedures were conducted by trained researchers in compliance with FELASA category C guidelines, and adhering to the European Union Directive (2010/63/EU) and Portuguese legislation (DL 113/2013) regarding the protection of animals used for scientific purposes (Decreto-Lei n.º 113/2013). The study protocol received approval from the Animal Welfare and Ethics Committee of the Interdisciplinary Centre of Marine and Environmental Research (ORBEA-CIIMAR).

2.2. Chemicals and experimental procedures

Sulfamethoxazole (SMX; CAS: 723–46–6, molecular weight 253.28 g/mol, ≥ 98 % purity) and trimethoprim (TRIM; CAS: 738–70–5, molecular weight 290.3 g/mol, ≥ 98.5 % purity) were purchased from Sigma Aldrich. To conduct the chronic assay, two stock solutions (100 mg/L for SMX and 50 mg/L for TRIM) were prepared by dilution of SMX and TRIM in dechlorinated tap water. The assay was performed for 28 days, according to OECD test guideline nº 215 (OECD, 2000), under the same laboratory-controlled conditions as those adopted during the quarantine period. The treatments tested were SMX (150 μg/L), TRIM (30 μg/L), an antibiotic mixture (MIX: 150 μg SMX/L + 30 μg TRIM/L), and a control group (without antibiotics). Each antibiotic treatment was tested at three different pH values (6.5, 7.5, and 9.0). The concentrations of SMX (150 μg/L) and TRIM (30 μg/L) used in this study were selected based on the highest levels reported in surface water monitoring studies. Kairigo et al. (2020) reported SMX concentrations of approximately 150 μg/L in surface waters impacted by wastewater discharge in Kenya, while Khan et al. (2013) detected TRIM concentrations of nearly 30 μg/L in rivers in Pakistan, also under strong anthropogenic influence. These concentrations, although representing worst-case scenarios, are environmentally relevant in regions with limited or no wastewater treatment. Thus, they provide a realistic basis for assessing potential ecological risks under high-exposure conditions. The pH values were chosen considering: the water laboratory system pH and standard guideline recommendations (7.5 – neutral pH), the IPCC predictions of freshwater acidification (pH = 6.5; IPCC 2023), and the alkaline freshwater projections in Portugal's freshwater ecosystems (pH = 9.0; INAG, 2011c, 2011b, 2011d, 2011a; Pinto et al., 2025; SNIRH, 2024).

A total of 216 *D. rerio* juveniles (1.58 ± 0.02 cm; 0.039 ± 0.004 g) were assigned to thirty-six 2-L glass aquaria randomly distributed in the exposure room (3 aquaria per treatment, each one with 6 fish). During the assay, fish were fed, and the exposure medium was 80 % renewed every 48 h. Physical and chemical water parameters (pH, temperature, conductivity, and dissolved oxygen) were measured using a multiparametric probe (Multi 3630 IDS SET F), and ammonium and nitrite

Table 1
Measured concentrations of the control group (CTL), sulfamethoxazole (SMX), trimethoprim (TRIM), and their mixture (MIX) in water samples collected at the beginning of the assay (0 h). Results of physical and chemical parameters monitored during chronic exposure, as well as water quality criteria under standard conditions based on OECD Guideline N°. 215 (OECD, 2000), were also presented.

Treatments		Measured concentrations (μg/L)	pH	Temp. (°C)	O_2 (%)	Nitrites (mg/L)	Ammonium (mg/L)
pH	Nominal μg/L)						
Estabilished quality criteria			6.5–8.5 ± 0.5	21–25 ± 2 °C	> 60 %		
Acidic pH 6.5	**CTL** (SMX = 0.0 TRIM = 0.0)	SMX = 0.0 TRIM = 0.0	6.61 ± 0.17	25.4 ± 0.36	95.9 ± 2.31	0.137 ± 0.02	0.44 ± 0.13
	SMX (150.0)	174.0	6.48 ± 0.05	25.8 ± 0.10	95.0 ± 3.05	0.239 ± 0.15	0.37 ± 0.01
	TRIM (30.0)	34.0	6.66 ± 0.17	25.4 ± 0.31	96.4 ± 1.22	0.125 ± 0.02	0.35 ± 0.21
	MIX (SMX = 150.0 TRIM = 30.0)	SMX = 200.0 TRIM = 32.3	6.60 ± 0.19	25.6 ± 0.15	96.2 ± 1.33	0.105 ± 0.01	0.52 ± 0.21
Neutral pH 7.5	**CTL** (SMX = 0.0 TRIM = 0.0)	SMX = 0.0 TRIM = 0.0	7.57 ± 0.06	25.6 ± 0.15	96.6 ± 2.91	0.317 ± 0.29	0.21 ± 0.25
	SMX (150.0)	144.0	7.55 ± 0.09	25.5 ± 0.25	96.2 ± 1.76	0.414 ± 0.15	0.15 ± 0.03
	TRIM (30.0)	32.5	7.52 ± 0.05	25.4 ± 0.20	97.0 ± 1.69	0.335 ± 0.36	0.19 ± 0.05
	MIX (SMX = 150.0 TRIM = 30.0)	SMX = 111.3 TRIM = 34.8	7.58 ± 0.05	25.8 ± 0.36	96.2 ± 1.27	0.336 ± 0.29	0.31 ± 0.33
Alkaline pH 9.0	**CTL** (SMX = 0.0 TRIM = 0.0)	SMX = 0.0 TRIM = 0.0	9.04 ± 0.18	25.7 ± 0.26	95.3 ± 2.34	0.408 ± 0.28	0.19 ± 0.08
	SMX (150.0)	114.0	9.09 ± 0.13	25.9 ± 0.15	96.3 ± 1.47	0.462 ± 0.36	0.28 ± 0.08
	TRIM (30.0)	31.0	9.01 ± 0.10	25.8 ± 0.36	96.0 ± 1.68	0.347 ± 0.25	0.29 ± 0.01
	MIX (SMX = 150.0 TRIM = 30.0)	SMX = 107.3 TRIM = 31.0	9.05 ± 0.23	25.5 ± 0.32	96.0 ± 1.38	0.449 ± 0.48	0.09 ± 0.04

levels were quantified using a bench photometer (Spectroquant Multy Colimeter) in aliquots of water collected from all aquariums before medium renewal (OECD, 2000).

2.3. Antibiotics quantifications

To quantify the analytical concentrations of SMX, TRIM, and MIX (Table 1), 50 mL of exposure medium was randomly collected from a replicate of each treatment at the beginning of the assay (0 h). The water samples were stored in darkness and frozen at −20 °C immediately after collection until the antibiotics were quantified. The analytical quantification followed the method described by Diogo et al. (2024). The limits of quantification (LOQs) were 1 μg/L for SMX and 0.8 μg/L for TRIM. The precision of the method was evaluated by examining its repeatability, and none of the compounds studied were detected in the control samples.

2.4. Sacrifice, biological samples collection, and biochemical markers

After 28 days (the exposure period), organisms were euthanized in a rapid ice-cold water bath (≤ 4 °C) and sacrificed by decapitation after no opercular movements and swimming mobility were observed, according to Portuguese animal welfare legislation and the American Veterinary Medical Association's recommendations for animal euthanasia (Decreto-Lei n.° 113/2013; Wilson et al., 2009). Then, the individuals were measured and weighed, and the biological samples were used to evaluate different biochemical markers. Thus, from each aquarium (replicate) the 6 fish were divided into: 2 fish bodies for oxidative stress and lipid peroxidation biomarkers determinations [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRed) and glutathione S-transferases (GSTs) activities, glutathione (GSH) content, thiobarbituric acid reactive substances (TBARS) levels]; 1 fish body for lactate dehydrogenase (LDH) activity; 1 body for cellular energy allocation [CEA - measuring the available energy (Ea), which includes total carbohydrate, lipid, and protein contents, as well as the energy consumed (Ec) through electron transport system (ETS) activity]; and 1 fish head was used for acetylcholinesterase (AChE) activity quantification. One fish head was immediately analyzed to assess DNA damage in the gills. All the biological samples were stored at −80 °C until the biochemical determinations follow the protocols described in Table 2.

2.5. Genotoxicity: DNA damage

Comet assay was used to evaluate the DNA damage and was performed according to Rodrigues et al. (2016). A six microgels system of 6 μL per slide/replicate was adopted to increase the assay output, based on a model created by Shaposhnikov et al. (2010) and described by Rodrigues et al. (2016). Microgels 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. DNA damage was assessed and quantified using a Nikon Eclipse Ci fluorescence microscope (600 × magnification), equipped with an excitation filter (540–580 nm) and an emission filter (620–670 nm). A total of 100 nucleoids per sample (i.e., replicate) were examined and classified into five categories (from 0 to 4) based on the intensity of the tail and head (Rodrigues et al., 2016). Cells from control organisms were treated with 50 μM of H_2O_2 for 5 min, as positive controls. The genetic damage index (GDI) was calculated according to Azqueta and Collins (2011), and results were expressed as arbitrary units on a scale of 0–400 per 100 scored nucleoids.

Table 2
Summary table of the procedures used for the biochemical determinations.

Biomarkers			Tissue preparation	Biomarker determinations		
			Homogenization Centrifugation	Spectrophotometric readings (nm)	Result expression units	References
SOD activity			2.5 mL - phosphate buffer (50 mM, pH 7.0) with Triton	500	units min /mg/protein	Flohé and Ötting (1984)
CAT activity			X−100 (0.1 %);	240	μmol/min/mg protein	Aebi (1984)
GPx activity			15,000 rpm; 10 min; 4 °C	340	mmol/min /mg protein	Flohé and Günzler (1984)
GRed activity				340	μmol/min/mg protein	Carlberg and Mannervik, 1985
GSH content				412	μg/mg protein	Soares et al., 2019
GSTs activity				340	mmol/min /mg protein	Habig et al., (1974)
TBARS levels				535	mmol/mg protein	Buege and Aust (1978)
CEA*	Ea	CARBO	1 mL of ice-cold phosphate buffer (50 mM, pH 7.0); 10,000 rpm; 5 min; 4 °C	492	mJ/mg fresh weight	De Coen and Janseen (1997)
		LIP		**		Folch et al., 1957
		PROT		595		Bradford (1976)
	Ec	ETS		490	mJ/mg fresh weight/ min	De Coen and Janseen (1997)
LDH activity			2.5 mL - TRIS buffer (0.1 M, pH=7.2): 6000 rpm; 3 min; 4 °C	340	mmol/min/mg protein	Vassault, 1983
AChE activity			1.5 mL - phosphate buffer (0.1 M, pH 7.2); 6000 rpm; 5 min; 4 °C	412	nmol/min/mg protein	Ellman et al., (1961)

*All the procedures were also present in Diogo et al (2025a). **Extraction procedure through the biphasic solvent system consisting of chloroform/methanol/water. The results (% of lipids) were obtained by the difference between the weight of tubes before and after the lipids extraction.

2.6. Data analysis

Data for all biomarkers were tested for normality (using the Shapiro-Wilk test) and homogeneity (using Levene's test). Before statistical analysis, the data for GRed activity, and GSH content activities were transformed (log(x) + 1 or arcsine) to meet ANOVA assumptions. A two-way ANOVA was conducted to evaluate the combined effects of antibiotics (SMX, TRIM, and MIX) and pH (6.5, 7.5, and 9.0) on *D. rerio*. To discriminate differences between antibiotic concentrations and the respective control treatment for each pH, a Dunnett's test was also performed. All statistical analyses were carried out with SPSS Statistics v29, using a significance level of $\alpha = 0.05$.

2.6.1. Ecotoxicological assessment

Regarding the biomarkers results, the effect percentage of each biomarker was calculated, for each antibiotic and pH condition, relative to the respective control group. This calculation followed the methodology described by Rodrigues et al. (2022) (Table S2), and established distinct ecotoxicity ranges (scores and classes). These results were used to assess the toxic effects of each antibiotic treatment under different pH conditions (Table S2).

2.6.2. IA model: SMX and TRIM potential interaction

To evaluate the potential interaction between SMX and TRIM in the MIX treatment under different pH scenarios (pH 6.5, 7.5, and 9.0), the Independent Action (IA) model was applied. The approach follows the conceptual basis established by Crain et al. (2008) and Piggott et al. (2015), and the specific methodology, including the equation and analytical steps, is described in Diogo et al. (2025b).

2.6.3. Biological health status of Danio rerio

Integrating multiple biomarker responses offers a more holistic understanding of the effects induced by different environmental stressors (Li et al., 2019). The biological health status of an organism exposed to different stressors can be evaluated through the biomarker response index (BRI), as proposed by Li et al. (2019). This index is determined by comparing the degree of alterations in biomarker responses in stressed organisms with the normal biological responses observed in a control group (without stress) (Li et al., 2019). In the BRI calculation, the relevance factor (W) for each biomarker was determined according to their biological significance and insights, proposed by Piva et al. (2011). According to Piva et al. (2011), this classification assigns relevance factors to biomarkers based on their ability to indicate adverse effects. These effects range from reversible responses (e.g., antioxidant defenses) to those signalling more severe biological damage (e.g., DNA damage). Thus, a W of 1.0 was applied to biomarkers of antioxidant defense, detoxification, and energetic metabolism (SOD, CAT, GRed, GPx, GSTs activities, GSH, and LDH contents), a W of 1.2 was assigned to biomarkers indicating potentially harmful effects (e.g., TBARS levels), and a W of 1.5 was reserved for biomarkers suggesting more severe damage (e.g., AChE activity and DNA damage). After that, the percentage of alterations (AL) observed in comparison to the respective control group was calculated:

$$AL\ (\%) = \frac{|BR_{antibiotic\ treatment} - \ BR_{CTL}|}{BR_{CTL}} \times 100$$

where $BR_{antibiotic\ treatment}$ refers to the result of each biomarker, and BR_{CTL} refers to the control group responses. According to AL (%) obtained, each biomarker response was classified into four different scores (1−4) (Table S2). Then, the BRI formula was applied:

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

where S_n were the score and W_n the relevance factor of biomarker n, respectively. Based on the calculated BRI, the biological health status of *D. rerio* was categorized as negligible, moderate, major, or severe alteration (Table S2; Hagger et al., 2008).

3. Results

3.1. Water quality

Throughout the chronic exposure, water quality parameters (pH, temperature, conductivity, dissolved oxygen, ammonium, and nitrites) remained within the established quality criteria (OECD, 2000), as detailed in Table 1. Measured concentrations for all antibiotic treatments are also provided in Table 1. No mortality was observed in the assays, complying with the guideline requirement of control group mortality being under 10 %.

3.2. Biochemical markers and genotoxicity results

The results of biochemical markers and DNA damage (genetic damage index) in *D. rerio* following exposure to environmentally relevant concentrations of SMX, TRIM, and their mixture (MIX) under different pH conditions (6.5, 7.5, and 9.0) are shown in Figs. 1–4. Antibiotics had significant effects on different biomarkers and DNA evaluation of *D. rerio* after chronic exposure, and significant interaction with pH, except in protein content, was observed (Figs. 1–4, and Table S1).

3.2.1. IPCC predictions of freshwater acidification: pH 6.5

Regarding antioxidant defense, no significant changes in SOD activity were observed after exposure to any antibiotic treatment at pH 6.5 (Fig. 1 and Table S1). At the same pH, CAT activity only decreased after TRIM exposure, while GRed activity increased significantly after SMX and MIX exposure. Similarly, GPx activity and GSH content increased after SMX exposure, whereas the opposite trend was observed following TRIM and MIX exposure (Fig. 1 and Table S1). GSTs activity significantly increased after exposure to SMX and MIX, while TBARS levels followed the same pattern, but decreased after TRIM exposure. A significant decrease in AChE and LDH activities was observed after exposure to all the antibiotic treatments. Carbohydrate content, Ea, and Ec increased following all antibiotic exposures (Fig. 1 and Table S1). In contrast, lipid content significantly decreased after exposure to all antibiotic treatments, while protein content showed no significant changes. CEA only decreased after SMX and MIX (Fig. 1 and Table S1).

Regarding the comet assay results, SMX exposure caused notable DNA damage, mainly classes 1 and 2, while TRIM led to genotoxic damage, predominantly classes 1, 2, but also 3. MIX treatment resulted in more severe damage, with damage classes 2, 3, and 4 most representative (Fig. 4I). All antibiotic treatments significantly increased the genetic damage index (Fig. 4II and Table S1).

Concerning IA model and the interaction obtained between SMX and TRIM in MIX treatment, an antagonistic down effect was observed for SOD, GPx and LDH activities, and GSH and protein contents (Fig. 1). In contrast, a synergistic up response was detected for CAT, GRed, and GSTs activities, TBARS levels, carbohydrate content, Ea (Fig. 1), and genetic damage index (Fig. 4II). AChE activity and lipid content showed an antagonistic up interaction (Fig. 1). Ec exhibited a synergistic down response. Finally, the CEA showed an additive effect (Fig. 1).

3.2.2. Neutral conditions: pH 7.5

The antioxidant defense and detoxification enzymes exhibited varied responses under neutral pH exposure (Fig. 2 and Table S1). A significant decrease in SOD activity was observed after exposure to TRIM and MIX, while CAT and GRed activity significantly increased after the same

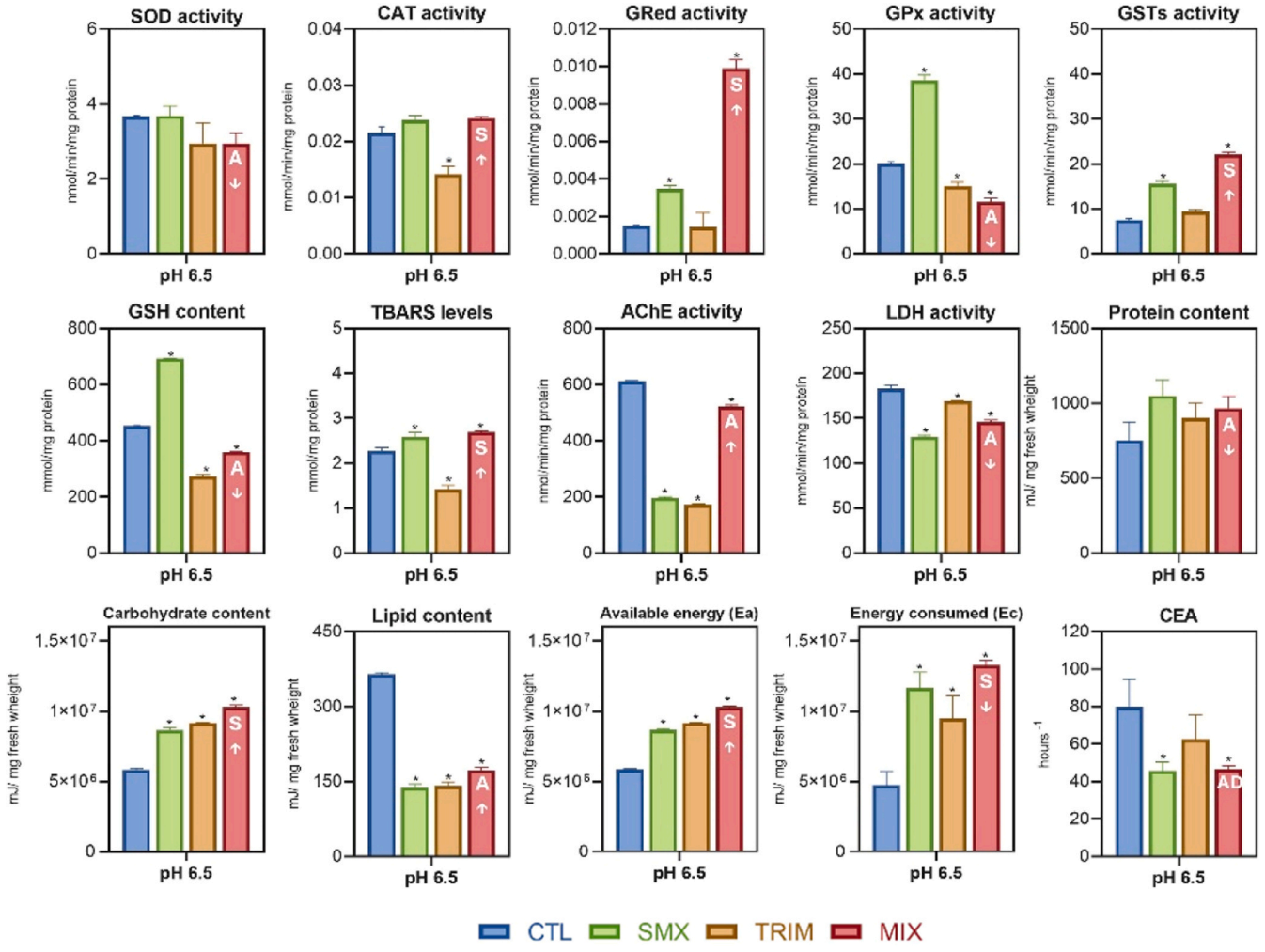

Fig. 1. Results of biochemical markers of *Danio rerio* after chronic exposure (28 days) to sulfamethoxazole (150 μg SMX/L), trimethoprim (30 μg TRIM/L), and a mixture (MIX = 150 μg SMX/L + 30 μg TRIM/L) under IPCC predictions of freshwater acidification (pH 6.5). Data are expressed as mean (n = 3) ± standard error bars. Asterisks (*) discriminate significant differences between the control group and antibiotic treatments (Dunnett's test; $p < 0.05$). The interaction type, determined using the Independent Action (IA) model and referring to the combined effects observed between the antibiotics SMX and TRIM in the MIX treatment, is also presented. AD – additive; S – synergism; A – antagonism; ↑ for up; ↓ for down.

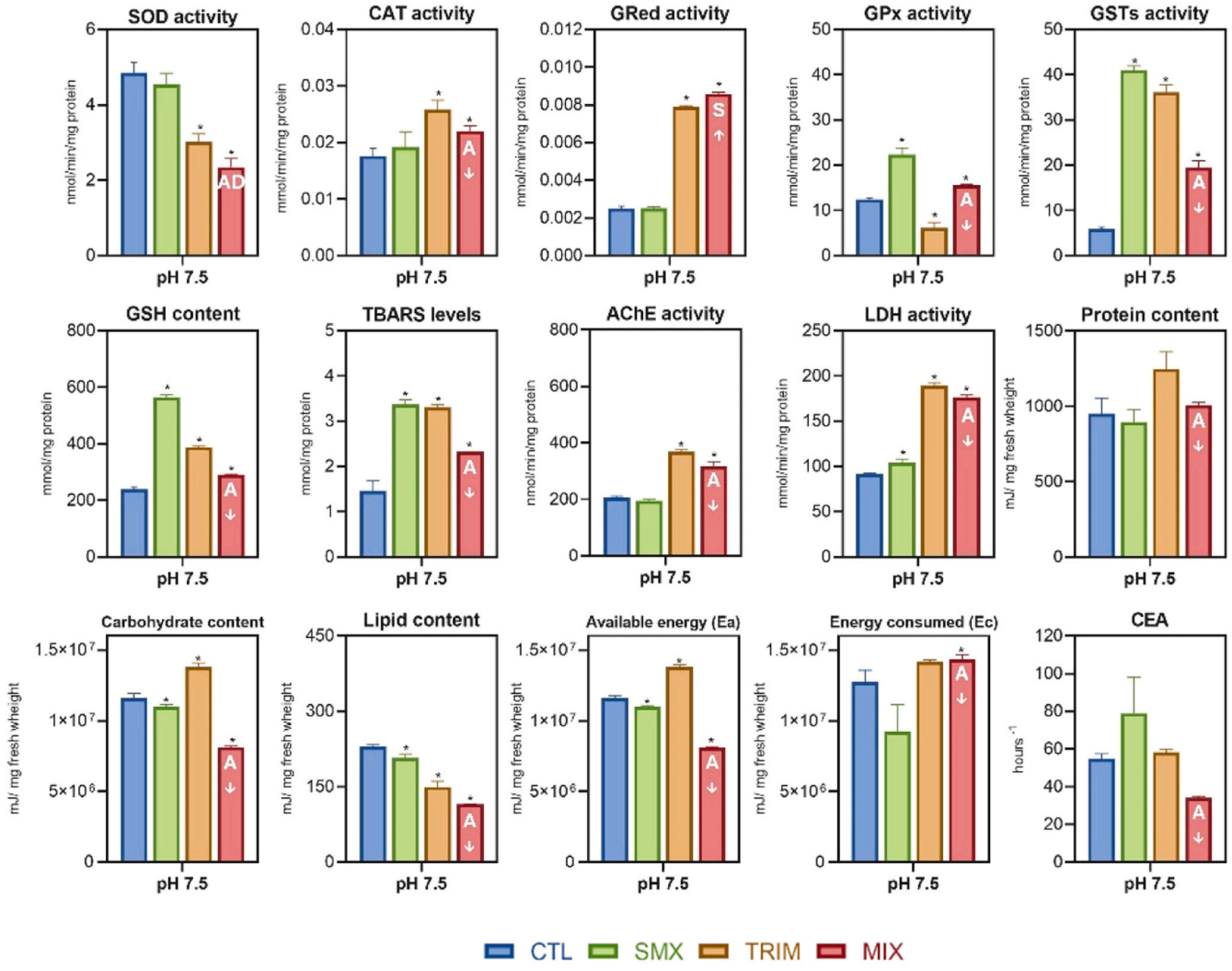

Fig. 2. Results of biochemical markers of *Danio rerio* after chronic exposure (28 days) to sulfamethoxazole (150 μg SMX/L), trimethoprim (30 μg TRIM/L), and a mixture (MIX = 150 μg SMX/L + 30 μg TRIM/L) under neutral conditions (pH 7.5). Data are expressed as mean (n = 3) ± standard error bars. Asterisks (*) discriminate significant differences between the control group and antibiotic treatments (Dunnett's test; $p < 0.05$). The interaction type, determined using the Independent Action (IA) model and referring to the combined effects observed between the antibiotics SMX and TRIM in the MIX treatment, is also presented. AD – additive; S – synergism; A – antagonism; ↑ for up; ↓ for down.

antibiotic treatments. GPx activity showed an increase after exposure to SMX and MIX but decreased following TRIM exposure (Fig. 2 and Table S1). A significant rise in GSTs activity, GSH, and TBARS levels across all antibiotic treatments was also observed. Relative to cholinergic neurotransmission, the results showed that AChE activity was significantly increased after exposure to TRIM and MIX (Fig. 2 and Table S1). Regarding energetic metabolism, LDH activity increased after exposure to all antibiotics (Fig. 2 and Table S1), while carbohydrate content and Ea decreased following SMX and MIX exposure, but increased with TRIM. In contrast, lipid content significantly decreased after exposure to all the antibiotic treatments, while no significant alterations were observed in protein content. Ec levels increased only after MIX exposure, while no significant changes were observed in CEA.

Based on the results from the comet assay, all antibiotic treatments led to a significant increase in the genetic damage index (Fig. 4II and Table S1). Organisms exposed to SMX and TRIM showed considerable DNA damage, particularly in classes 1 and 2 comets, while those exposed to MIX exhibited more severe damage, with DNA damage predominantly in classes 2, 3, and 4 (Fig. 4I).

Using the IA model, an additive effect was observed for SOD activity, while a synergistic increase was detected for GRed activity (Fig. 2) and genetic damage index (Fig. 4II) following MIX exposure. In contrast, the remaining parameters exhibited an antagonistic down response (Fig. 2).

3.2.3. *Alkaline freshwater projections: pH 9.0*

Under alkaline pH, no significant changes were observed in SOD activity, while a significant increase was recorded in CAT activity and GSH content, after exposure to SMX and TRIM (Fig. 3 and Table S1). A significant increase was also observed in GRed and GSTs activities, and TBARS levels after TRIM and MIX exposure, and in GPx activity only after SMX exposure (Fig. 3 and Table S1). Regarding AChE activity, a significant decrease was observed after SMX exposure, while the opposite was observed in TRIM and MIX exposure. LDH activity was significantly increased following exposure to all the antibiotic treatments (Fig. 3 and Table S1). A significant decrease in carbohydrate content, lipid content, Ea, and CEA was observed after exposure to all the antibiotic treatments, while protein and Ec remained unchanged.

Considering the results obtained in the comet assay, a significant increase in the genetic damage index was observed in all the antibiotic treatments (Fig. 4II and Table S1). However, overall, the organisms exposed to SMX exhibited a higher percentage of severe DNA damage (classes 3 and 4) than TRIM and MIX (less severe, classes 2 and 3).

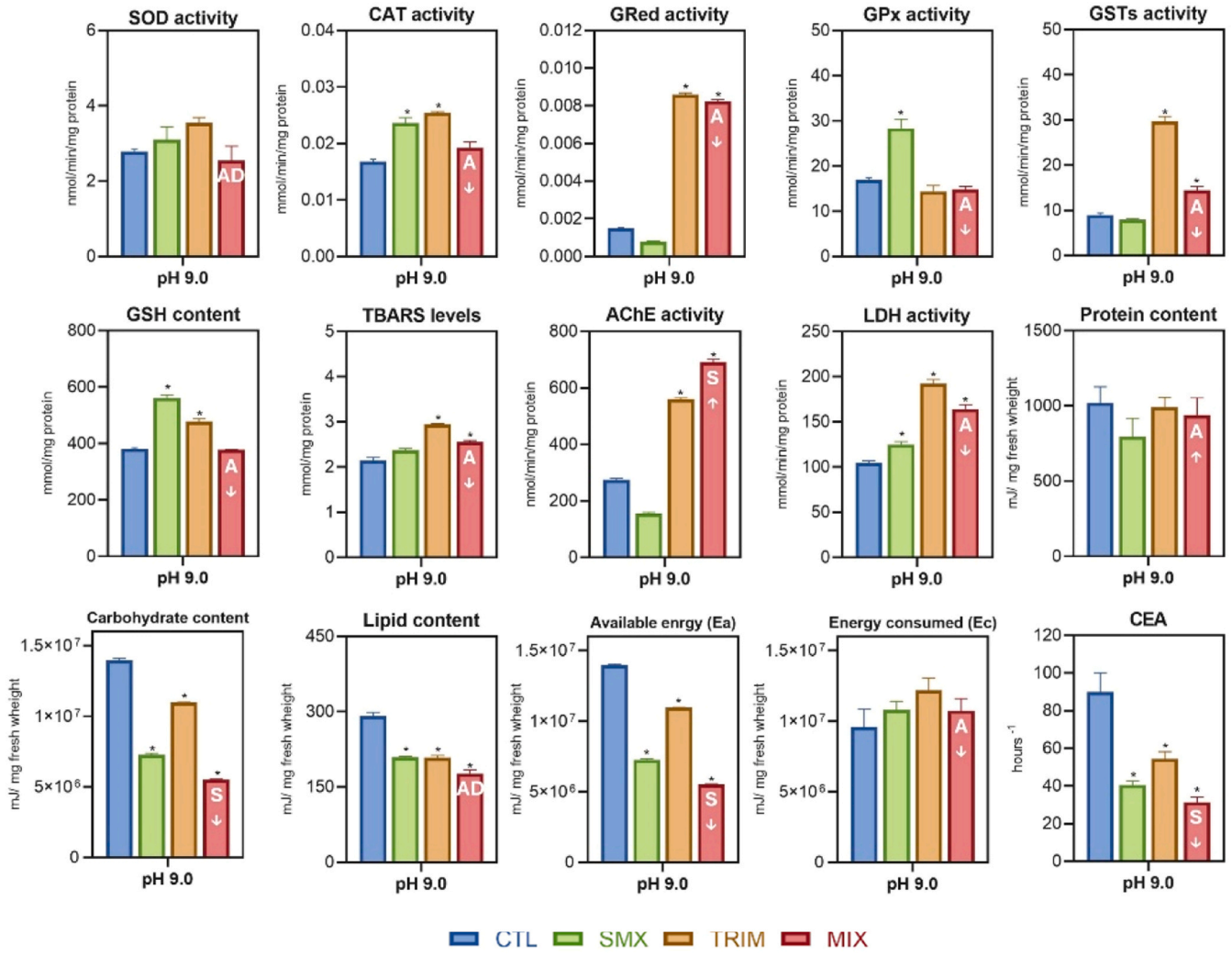

Fig. 3. Results of biochemical markers of *Danio rerio* after chronic exposure (28 days) to sulfamethoxazole (150 μg SMX/L), trimethoprim (30 μg TRIM/L), and a mixture (MIX = 150 μg SMX/L + 30 μg TRIM/L) under alkaline freshwater projections (pH 9.0). Data are expressed as mean (n = 3) ± standard error bars. Asterisks (*) discriminate significant differences between the control group and antibiotic treatments (Dunnett's test; $p < 0.05$). The interaction type, determined using the Independent Action (IA) model and referring to the combined effects observed between the antibiotics SMX and TRIM in the MIX treatment, is also presented. AD – additive; S – synergism; A – antagonism; ↑ for up; ↓ for down.

I)

Treatments		Damage classes				
pH	Antibiotic	0	1	2	3	4
Acidic pH 6.5	CTL	60.3 ± 5.4	37.0 ± 5.1	2.70 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
	SMX	12.3 ± 0.0	68.3 ± 0.7	19.3 ± 0.4	0.0 ± 0.0	0.0 ± 0.0
	TRIM	6.00 ± 0.0	60.0 ± 0.6	31.3 ± 0.6	2.70 ± 0.1	0.0 ± 0.0
	MIX	0.0 ± 0.0	5.30 ± 0.1	46.7 ± 0.9	41.0 ± 1.2	7.00 ± 0.3
Neutral pH 7.5	CTL	46.7 ± 4.1	53.3 ± 4.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	SMX	6.00 ± 0.0	75.7 ± 0.8	18.3 ± 0.4	0.0 ± 0.0	0.0 ± 0.0
	TRIM	4.70 ± 0.0	66.7 ± 0.7	28.0 ± 0.6	0.70 ± 0.0	0.0 ± 0.0
	MIX	0.0 ± 0.0	10.7 ± 0.1	67.0 ± 1.3	20.7 ± 0.6	1.7 ± 0.1
Alkaline pH 9.0	CTL	42.0 ± 2.0	53.7 ± 2.4	4.30 ± 0.7	0.0 ± 0.0	0.0 ± 0.0
	SMX	0.0 ± 0.0	4.70 ± 0.0	17.0 ± 0.3	53.7 ± 1.6	24.7 ± 1.0
	TRIM	0.0 ± 0.0	2.00 ± 0.0	32.0 ± 0.6	60.7 ± 1.8	5.30 ± 0.2
	MIX	0.0 ± 0.0	3.30 ± 0.0	64.7 ± 1.3	25.3 ± 0.8	6.70 ± 0.3

II)

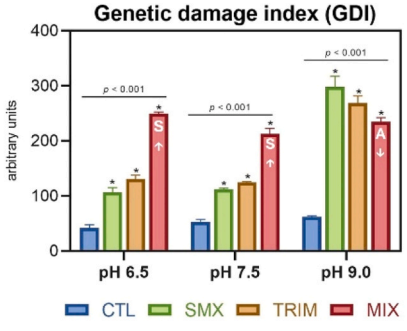

Fig. 4. Results of comet assay in gills of *Danio rerio* after chronic exposure (28 days) to sulfamethoxazole (150 μg SMX/L), trimethoprim (30 μg TRIM/L), and a mixture (MIX = 150 μg SMX/L + 30 μg TRIM/L). I) Percentage of damage classes (0−4); II) Results of Genetic Damage Index (GDI, expressed as arbitrary units). Data are expressed as mean ± standard error. Significant effects (p level) of antibiotics for each pH condition are shown, with asterisks (*) discriminating significant differences between the control group and antibiotic treatments in each pH value (Dunnett's test; $p < 0.05$). The interaction type, determined using the Independent Action (IA) model and referring to the combined effects observed between the antibiotics SMX and TRIM in the MIX treatment, is also presented. AD – additive; S – synergism; A – antagonism; ↑ for up; ↓ for down.

Regarding IA model, an additive effect was observed for SOD activity and lipid content after MIX exposure (Fig. 3). A synergism up response was detected for AChE activity, while a synergism down effect was observed for carbohydrate content, Ea, and CEA. For the remaining parameters, an antagonism down response was found (Fig. 3 and 4II).

3.3. Ecotoxicological assessment and Danio rerio biological health status

Table 3 shows the percentage effects of each biomarker (Table 3. I) evaluated in *D. rerio* after exposure to environmentally relevant concentrations of SMX, TRIM, and their MIX under different pH conditions (6.5, 7.5, and 9.0), and the final toxicity classification obtained (Table 3. I.a). The BRI values (Table 2. II) obtained (Table 3 and Table S2) and the corresponding biological health status were also presented. At pH 6.5, the results showed that SMX and MIX were classified as marginally toxic (score 3), while TRIM was classified as slightly toxic (score 2). Regarding the BRI, SMX caused severe alterations ($1.00 < BRI < 2.50$) in *D. rerio* health status, while TRIM and MIX only caused major alterations (BRI ranging from 2.51 to 2.75; Table 2). At neutral pH (7.5), both SMX and TRIM were classified as marginally toxic, and caused major and severe alterations in the health status of *D. rerio* (respectively), while MIX was classified as moderately toxic (score 4) and induced severe alterations in this species (Table 3). All antibiotic treatments revealed marginal toxicity after exposure to alkaline pH (9.0), and TRIM and MIX exposure caused severe alterations in *D. rerio's* health status, while SMX only caused moderate alterations (Table 3).

4. Discussion

pH is a fundamental abiotic factor in aquatic ecosystems, directly influencing the physiology of organisms and the chemical balance of the environment (Suter et al., 2023). Small pH variations can influence nutrient availability, cell membrane permeability (e.g., the presence of transporters, the type of transport involved, the electrochemical gradient), and the toxicity of chemical compounds in the environment (AlRabiah et al., 2018; Sun et al., 2020). pH plays a crucial role in the toxicity of a compound, influencing its solubility, chemical stability, bioavailability, and, in turn, toxicity, either enhancing or decreasing the impact (AlRabiah et al., 2018; Paul et al., 2020; Sun et al., 2020; Suter et al., 2023). The sensitivity of organisms to these compounds is also strongly influenced by pH fluctuations, making this factor crucial for assessing the ecotoxicological impact of pollutants in aquatic environments (Anskjær et al., 2013; Sun et al., 2020; Valenti et al., 2009). However, studies that examine these interactions in freshwater

Table 3

I) The percentage effects in each biomarker of *Danio rerio* following chronic exposure to environmentally relevant concentrations of sulfamethoxazole (150 μg SMX/L), trimethoprim (30 μg TRIM/L), and their mixture (MIX = 150 μg SMX/L + 30 μg TRIM/L) were assessed under varying pH conditions (6.5, 7.5, and 9.0) (Table S2). I.a) Final toxicity: scores (1–5) and the respective final ecotoxicological classification [non toxic (NT – blue), slightly toxic (ST - green), marginally toxic (MGT - yellow), moderately toxic (MT - orange), highly toxic (HT – red)] determined for each antibiotic treatment (Table S2). II) Results of Biomarker Response Index (BRI) and the corresponding classification of biological health status. BRI values: $1.00 \leq BRI \leq 2.50$ - severe alterations (red); $2.51 \leq BRI \leq 2.75$ - major alterations (orange); $2.76 \leq BRI \leq 3.00$ - moderate alterations (yellow); $3.01 \leq BRI \leq 4.00$ - negligible alterations (green) (more details in Table S2).

Biomarker	I) Effects (%)								
	Acidic pH 6.5			Neutral pH 7.5			Alkaline pH 9.0		
	SMX	TRIM	MIX	SMX	TRIM	MIX	SMX	TRIM	MIX
SOD activity	0.40	-19.7	-20.0	-6.35	-38.0	-51.6	12.0	27.9	-7.80
CAT activity	10.8	-34.0	12.5	8.77	48.8	24.9	41.0	51.8	15.0
GRed activity	132	-4.70	563	0.05	215.4	242.7	-47.4	473	449
GPx activity	92.2	-24.8	-42.8	132.0	-4.74	563.3	66.8	-15.2	-12.4
GSH content	52.7	-39.5	-20.7	135.5	61.4	21.2	47.3	25.5	-0.90
GSTs activity	107	25.7	195	595.3	512.5	231.3	-11.8	232	60.8
TBARS levels	13.6	-37.3	18.1	130.8	126.2	59.3	10.7	37.4	19.2
AChE activity	-67.8	-71.6	-14.6	-5.51	78.2	53.8	-43.5	103	151
LDH activity	-29.2	-7.50	-20.1	13.8	106.1	92.1	19.5	83.5	56.8
CEA	-43.1	-21.8	-41.6	43.6	6.12	-38.2	-54.9	-39.4	-65.2
GDI	153	209	490	111	134	300	379	332	278
I.a) Final toxicity	**3 MGT**	**2 ST**	**3 MGT**	**3 MGT**	**3 MGT**	**4 MT**	**3 MGT**	**3 MGT**	**3 MGT**
II) Biomarker Response Index									
BRI	**2.40**	**2.80**	**2.73**	**2.60**	**1.86**	**2.04**	**2.85**	**2.10**	**2.43**
Biological Health Status	**Severe**	**Major**	**Major**	**Major**	**Severe**	**Severe**	**Moderate**	**Severe**	**Severe**

organisms remain limited, since pH change has often been associated only with acidification in seawater ecosystems (Almeida et al., 2022; Bethke et al., 2023; Jesus et al., 2018).

Several studies have reported that the interaction between pharmaceuticals (e.g., antibiotics, antiepileptics) and climate change factors (e.g., pH fluctuations) can either enhance or attenuate the effects of these compounds (e.g., Alsop and Wilson, 2019; Bethke et al., 2023; Nakamura et al., 2008; Valenti et al., 2009). The toxicity of pharmaceuticals is directly influenced by pH, which regulates the balance between the charged and neutral forms of pharmaceutical molecules by altering the charges of the acidic and alkaline parts of the molecules (uncharged or neutral molecules, in turn, pass through cell membranes more readily) (Alsop and Wilson, 2019; Bethke et al., 2023). Studies have shown that more than 80 % of these compounds are ionizable, meaning their toxicity can vary depending on whether the neutral or ionized form predominates under different pH conditions (Manallack, 2007). Typically, the neutral form is more lipophilic and, therefore, has a greater capacity to cross cell membranes, often making it the primary contributor to a compound's toxicity (Valenti et al., 2009). This is because, for pharmaceuticals to be effective, they must be designed to penetrate cells, a process influenced by the molecule's hydrophobicity and acidity (Almeida et al., 2022; Kim et al., 2010). In general, pH influences a compound's toxicity and can modify its absorption by organisms. It can also affect the stability of toxic substances and compromise cellular processes, such as membrane permeability and enzymatic activity. Furthermore, pH can enhance or reduce the toxicity of contaminants in the environment, making its control essential in assessing chemical risks.

4.1. Toxicity of sulfamethoxazole under pH variations

The toxicity of SMX in *D. rerio* varied with pH, influencing physiological and biochemical responses, including antioxidant defenses, metabolic processes, and overall well-being (Fig. 5). At acidic (6.5; Figs. 1 and 5) and neutral (7.5; Figs. 2 and 5) pH, SMX increased the activity of several antioxidant enzymes, lipid peroxidation, and genotoxicity, suggesting that this antibiotic may induce oxidative stress and cellular damage in *D. rerio* (Figs. 4 and 5). The opposite was observed after exposure to SMX at alkaline pH (9.0; Figs. 3 and 5), indicating that *D. rerio* may be activating different mechanisms (e.g., antioxidant and detoxification mechanisms) to cope with the induced oxidative stress, managing to prevent lipid peroxidation (Fig. 3), but not genotoxicity (Fig. 4). The defense responses may prioritize protecting cellular membranes from oxidative damage (lipid peroxidation), but nuclear material may remain exposed (membrane stability *vs*. nuclear vulnerability), leading to genotoxic effects. Thus, at pH 9.0, there was a less toxic profile, possibly due to lower solubility or ionization of the compound that limits its passive diffusion through membranes (Fig. 5).

Anskjær et al. (2013) studied the acute toxicity of sulfadiazine in *Daphnia magna*, in three pH conditions (6.0, 7.5, and 8.5), reporting a significant increase in toxicity with decreasing pH. These authors explained these results considering that possibly the greatest amount of the non-ionized (neutral) fraction of the antibiotic was greater at the lowest pH (6.0), resulting in greater diffusion through the cell membranes of *D. magna* (Anskjær et al., 2013). The same tendency was observed in the present study since SMX is a sulfonamide antibiotic classified as a weak acid, whose ionization depends heavily on the environmental pH (Bethke et al., 2023; Montone et al., 2024). At lower pH values, a larger proportion of molecules remain in their non-ionized form, while at higher pH values, they become more ionized (Montone et al., 2024). At pH 6.5, most of the SMX is expected to be in the neutral form, which is more lipophilic and can more easily cross cell membranes via passive diffusion (Fig. 5). Our results also suggest that the acidic environment potentiates the active or bioavailable form of SMX, increasing its ability to cause cellular damage. As a result, SMX is likely to be more toxic at low pH values due to increased intracellular accumulation, heightening its toxic effects (Bethke et al., 2023). Similar results were reported by Paul et al. (2020), who studied the toxicity of triclosan (also classified as a weak acid) in the fish *Pangasianodon hypophthalmus* at different pH levels (6.5, 7.5, and 8.5). These authors found that increased toxicity at lower pH levels was attributed to the formation of the non-ionized triclosan, which can more easily diffuse through gills and skin, resulting in higher bioavailability and toxicity (Paul et al., 2020). Furthermore, several studies have reported that

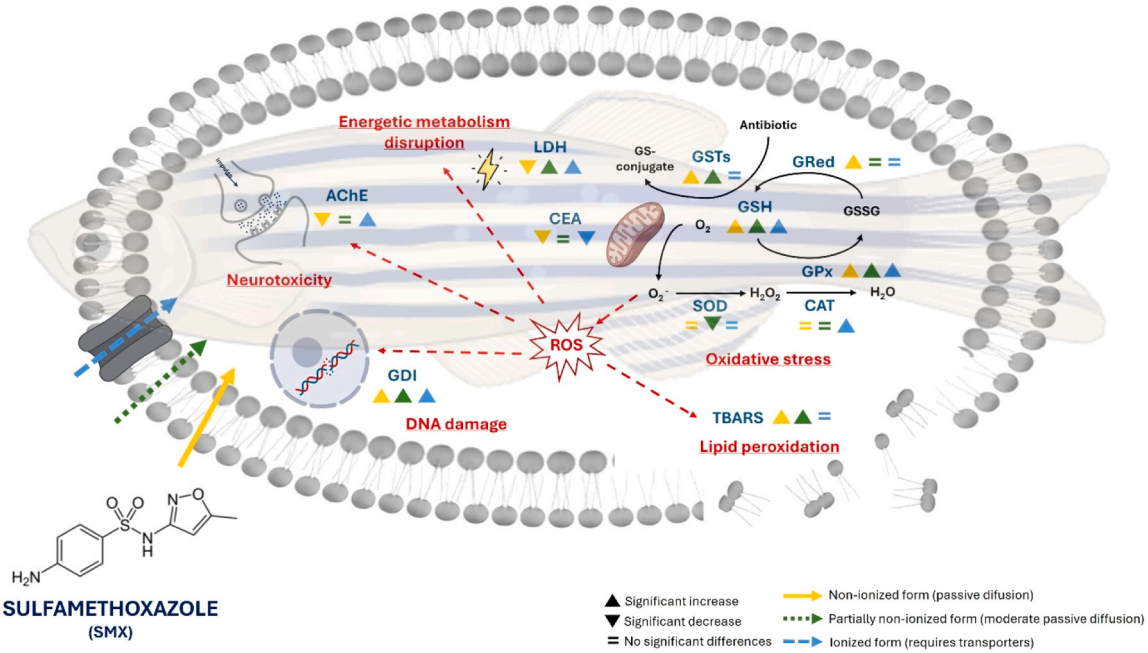

Fig. 5. Description of the mechanisms of toxicity for sulfamethoxazole (SMX) across different pH conditions (6.5, 7.5, and 9.0), according to the results of biochemical responses, illustrating how pH influences SMX toxicity. Key pathways and effects are color-coded by pH condition: yellow for pH 6.5, green for pH 7.5, and blue for pH 9.0, highlighting variations in toxicity mechanisms across the tested pH range.

pharmaceuticals classified as weak acids (e.g., triclosan, naproxen, diclofenac, salicylic acid) can alter biomarkers associated with antioxidant and biotransformation defense mechanisms (e.g., SOD, CAT, GPx, and GSTs), as well as indicators of cellular damage (TBARS levels), through their ability to affect the redox balance of the organisms (e.g., Almeida et al., 2022; Costa et al., 2020; Munari et al., 2018). Costa et al. (2020) investigated the biochemical and physiological responses of clams *Ruditapes philippinarum* and *Ruditapes decussatus* under exposure to triclosan and different pH conditions (pH 7.1 and 8.1). The study showed that oxidative stress responses and lipid peroxidation were affected by pH variations, with *R. decussatus* exhibiting higher cellular damage (Costa et al., 2020). Although no significant changes in SOD activity were observed across pH conditions, Costa et al. (2020) observed that CAT and GSTs activities increased significantly under the combined stressors, suggesting activation of antioxidant and detoxification mechanisms to cope with oxidative stress and other physiological disruptions. Munari et al. (2018) studied the biochemical responses of *Mytilus galloprovincialis* under reduced pH conditions and diclofenac exposure. The authors revealed that, at low pH (−0.4 and −0.7 units from the standard pH = 8.1), the activities of SOD and CAT remained unchanged, suggesting that other antioxidant mechanisms might be responsible for mitigating oxidative stress under these conditions (Munari et al., 2018). Additionally, increased DNA strand breaks were observed in the gills in low pH and diclofenac exposure (Munari et al., 2018). Dionísio et al. (2020) investigated the toxicity of salicylic acid in *Gibbula umbilicalis*, exposed to three environmentally relevant concentrations (5, 25, and 125 μg/L) and different pH conditions (7.6, 7.9, and 8.2). These authors observed a significant effect of pH on cyclooxygenase, GSTs, and AChE activities, with changes dependent on both pH and salicylic acid concentrations. The results suggest that changes in pH values from normal conditions may contribute to oxidative stress or increased oxidative damage in exposed organisms (Dionísio et al., 2020).

Exposure to antibiotics in changing pH scenarios also represents a potentially disruptive combination for the energetic systems of aquatic organisms (Bethke et al., 2023; Jesus et al., 2018). To survive challenging conditions, organisms often display various physiological responses, including adjustments in metabolic pathways that redirect energy production to cope with stress (Jesus et al., 2018; Shang et al., 2023). The exposure of *D. rerio* to acidic pH and SMX significantly reduces CEA, reflecting a disruption in the balance between energy needs and availability (Figs. 1 and 5). Additionally, a significant decrease in LDH activity suggests a diminished reliance on anaerobic metabolic pathways, further highlighting disruptions in the organism's overall energy metabolism. This aligns with findings from other studies, such as Shang et al. (2023), which also observed a decrease in CEA in mussels (*Mytilus coruscus*) under acidification, due to an energy imbalance. Almeida et al. (2022) investigated the effects of cetirizine and carbamazepine, both individually and in a mixture, on *R. philippinarum* under acidic pH conditions and found significant impacts on energy metabolism. This study revealed that exposure to a lowered pH, in combination with these pharmaceuticals, notably increased the Ec, likely linked to enhanced detoxification processes, to eliminate reactive oxygen species generated under stress conditions (Almeida et al., 2022). Typically, LDH activity increases under stressful conditions (e.g., high temperatures, scarcity of oxygen), as observed by Jesus et al. (2018) in freshwater fish *Squalius carolitertii* under an acidification scenario (pH 6.5–6.9). However, in the present study, exposure to SMX led to a similar response, but at neutral and alkaline pH (7.5 and 9.0; Figs. 2, 3, and 5). These findings, consistent with the significant effects observed in Ea and Ec, suggest that *D. rerio* may shift its energy metabolism toward anaerobic pathways to support cellular activity, under neutral and alkaline pH (Figs. 2 and 3, respectively). This indicates potential energy balance disruptions in response to the combined SMX and pH stress (Farhana and Lappin, 2024). Costa et al. (2020) studied the effects of weak acid (triclosan) and pH on clams *R. philippinarum* and *R. decussatus*, finding decreased Ea (protein and lipid levels) at pH 7.7 without impacting Ec, similar to our results. Furthermore, Bolner et al. (2014) observed that under alkaline pH conditions, glucose and glycogen levels in silver catfish (*Rhamdia quelen*) decreased significantly, suggesting that the species have fewer available energy reserves. This decline may compromise key physiological processes essential for fish homeostasis, development, and survival (Bolner et al., 2014).

Another physiological process that can be influenced by pH variations and affect the health of the organism is neurotransmission (Marinho et al., 2019; Serova et al., 2020). A significant decrease in AChE activity after exposure to SMX at pH 6.5 (Figs. 1 and 5) and 9.0 (Figs. 3 and 5) was observed, while no significant changes occurred at neutral pH (Figs. 2 and 5). In contrast to our results, other authors reported a significant variation in the AChE activity of *D. rerio* embryos after exposure to SMX (> 40 μg/L), enrofloxacin (6 and 60 μg/L), and norfloxacin (> 200 μg/L) at neutral pH (Liu et al., 2014; Tian et al., 2024). Marinho et al. (2019) reported that AChE activity is highly sensitive to pH levels, with the optimal pH for brain AChE in *D. rerio* being 9.0 and for muscle AChE being 8.5 (Ceylan and Erdogan, 2017). Both brain and muscle AChE show a decrease in activity at extreme pH levels, likely due to protonation effects that alter the active site's properties, which disrupt the enzyme-substrate interaction (de la Torre et al., 2002; Marinho et al., 2019). Specifically, increased protonation of histidine residues in the AChE active site at acidic pH hinders its ability to interact with the substrate, decreasing enzymatic activity (Marinho et al., 2019). Several authors have reported the impact of different antibiotics (such as cefalexin, sulfadiazine, norfloxacin, and oxytetracycline) on AChE activity in fish, which interferes with normal cholinergic neurotransmission and may lead to adverse effects (e.g., visual difficulties, cognitive impairment, and impaired movement coordination) (Huo et al., 2023; Miranda et al., 2019).

4.2. Toxicity of trimethoprim under pH variations

Under all the pH conditions, TRIM exposure activated antioxidant defenses and detoxification processes in *D. rerio*, however, these mechanisms were insufficient to prevent lipid peroxidation under neutral (Figs. 2 and 6) and alkaline pH (Figs. 3 and 6). This suggests that while TRIM exposure triggered defense responses, these were not always effective in mitigating cellular damage, particularly at neutral and alkaline pH. Additionally, genotoxicity was also observed in all pH conditions, indicating that TRIM exposure consistently led to DNA damage regardless of the pH (Figs. 4 and 6), and can still occur if repair mechanisms are insufficient or overwhelmed. DNA is particularly vulnerable to oxidative stress, and some oxidative byproducts may persist despite cellular defenses. Unlike SMX, TRIM is a weak base, and its ionization behavior is inversely related to pH (AlRabiah et al., 2018). Different authors (e.g., Nakamura et al., 2008; Valenti et al., 2009) reported that the toxicity of some pharmaceuticals classified as weak bases (e.g., fluoxetine, sertraline, carbamazepine) increased with pH, indicating that, in more neutral and alkaline conditions, a greater proportion of the molecules remain in the non-ionized form (facilitating their passage through cell membranes and consequently increasing absorption and toxicity in the aquatic organisms studied; Fig. 6).

Nakamura et al. (2008) reported that the sensitivity of fish *Oryzias latipes* to fluoxetine varied significantly at different pH levels, concluding that higher pH values lead to increased toxicity. These authors revealed that LC_{50} values after 96 h decreased from 5.5 mg/L at pH 7 to 0.20 mg/L at pH 9, indicating that fish are more vulnerable to fluoxetine at alkaline pH levels. Additionally, the bioconcentration of fluoxetine increased at high pH, corroborating the idea that the molecule's neutrality favors its accumulation in the tissues of *Oryzias latipes*. Similarly, Valenti et al. (2009) studied the sertraline toxicity in fish *Pimephales promelas* at different pH levels (6.5, 7.5, and 8.5), reporting that lower concentrations of this compound (< 1000 μg/L) caused notable toxicity (e.g., mortality, decrease in growth and feeding rates) at

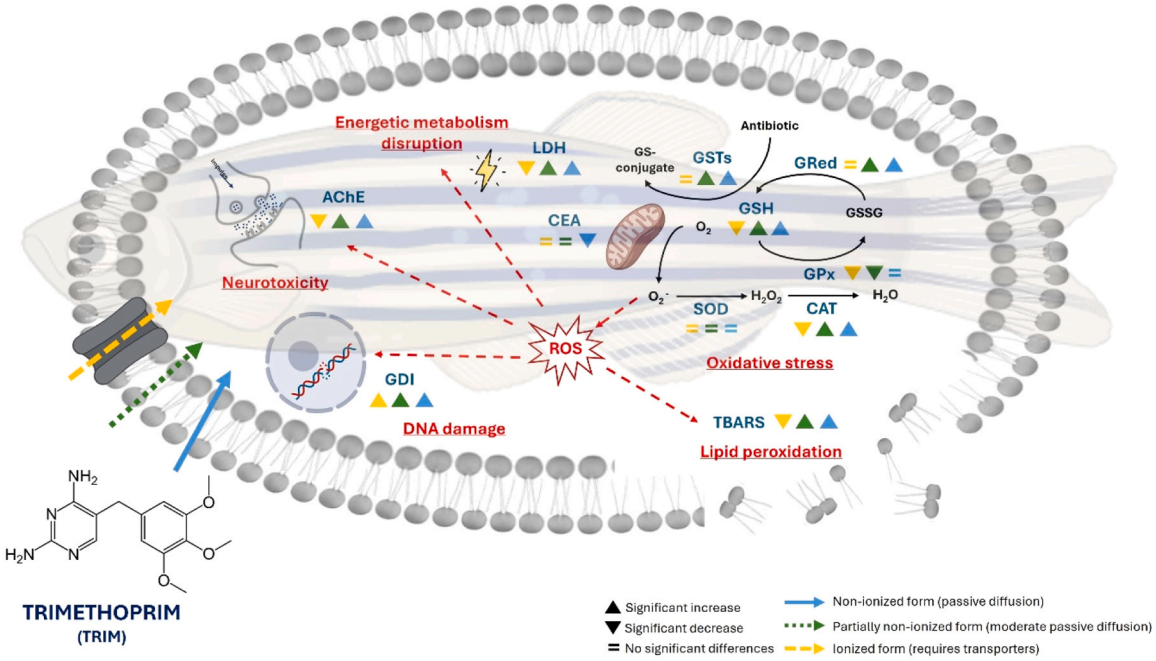

Fig. 6. Description of the mechanisms of toxicity for trimethoprim (TRIM) across different pH conditions (6.5, 7.5, and 9.0), according to the results of biochemical responses, illustrating how pH influences SMX toxicity. Key pathways and effects are color-coded by pH condition: yellow for pH 6.5, green for pH 7.5, and blue for pH 9.0, highlighting variations in toxicity mechanisms across the tested pH range.

pH 8.5, compared to pH 6.5 and 7.5. These authors highlighted that the ionization state of sertraline, which fluctuates with pH, directly influences its toxicity (Valenti et al., 2009). At higher pH levels, sertraline is more often in its non-ionized form, which is more readily absorbed by organisms, resulting in a quicker onset of adverse effects (Valenti et al., 2009). At more acidic pH levels, such as 6.5, TRIM exists in an ionized form, which can hinder its ability to pass through cell membranes since charged molecules are less likely to diffuse passively across lipid membranes (Fig. 6), thereby reducing their toxicity (AlRabiah et al., 2018; Straub, 2013). As the pH increases, such as at 7.5 or 9.0, a larger fraction of TRIM is found in the non-ionized (neutral) form, which enhances its diffusion across cell membranes (AlRabiah et al., 2018; Straub, 2013). Consequently, at these pH levels, TRIM is likely to be more readily absorbed by organisms, potentially enhancing their toxicity, as demonstrated in this study (Figs. 2 and 3). Mikes and Trapp (2010) studied the toxicity of TRIM in basket willow (*Salix viminalis*) and found that TRIM's toxicity levels vary depending on the environment pH. These authors also reported that TRIM toxicity was higher at high pH (pH 8–9) compared to low pH (pH 4.3). Additionally, these authors suggest that the toxicity and bioaccumulation of ionizable compounds like TRIM are likely to be more pronounced when the compound is in its neutral form, which occurs at higher pH levels (Mikes and Trapp, 2010). Almeida et al. (2022) investigated the effects of environmentally relevant concentrations of two weak bases, carbamazepine (1 μg/L) and cetirizine (0.6 μg/L), on the activity of antioxidant enzymes in clams (*R. philippinarum*). The study found that clams exposed to both compounds at lowered pH (-0.4 units from the standard pH = 8.0) exhibited higher activity levels of antioxidant and biotransformation enzymes, such as CAT and GSTs. These findings suggest that lower pH conditions may enhance processes for eliminating reactive oxygen species (ROS) in these organisms (Almeida et al., 2022).

In line with this, under acidic pH, TRIM exposure induced metabolic stress in *D. rerio*, leading to increased energy consumption and activation of detoxification mechanisms (Figs. 1 and 6). However, despite these responses, overall energy efficiency remained unchanged, and there was no shift toward anaerobic metabolism (Figs. 1 and 6). In contrast, at neutral pH, TRIM also impacted energy metabolism, affecting energy reserves without compromising balance, while the increase in LDH activity indicates a shift toward anaerobic pathways (Figs. 2 and 6). Pimentel et al. (2019) reported that LDH activity in *Sparus aurata* larvae significantly increased at pH 7.5, highlighting a shift toward anaerobic metabolism. This study also found a decrease in the activity of key aerobic enzymes (e.g., citrate synthase - CS), indicating a reduced aerobic capacity (Pimentel et al., 2019). This increase in LDH activity and the LDH/CS ratio suggests a necessary shift from aerobic to anaerobic energy production, enabling *S. aurata* larvae to maintain energy levels in response to environmental stress. The same tendency is observed in *Crassostrea gigas* (pacific oysters), which can modulate energy sources, inhibiting aerobic energy metabolism, after 28 days of exposure to pH 7.6 (Cao et al., 2018). Similar to the findings with SMX, in the alkaline pH, after TRIM exposure, LDH activity increased, suggesting a shift toward anaerobic metabolism (Figs. 3 and 6). This, along with the observed metabolic adjustments (alterations in Ea and CEA), indicates that the combined effect of TRIM and alkaline conditions causes a further disruption in the energy balance of *D. rerio*, potentially challenging its ability to maintain homeostasis (Fig. 3). As previously mentioned by Bolner et al. (2014), alkaline conditions increased Na^+/K^+-ATPase activity in the gills and kidneys of catfish *R. quelen*, directing more energy to ion regulation and leaving less energy available for other physiological functions (e.g., growth). This enzyme plays a crucial role in ion regulation and can contribute to maintaining higher pH levels within the fish's body (Bolner et al., 2014).

Although no studies have been found relating the combined effect of TRIM and pH variations on fish neurotransmission, it is known that AChE activity can be individually affected by antibiotics and water physicochemical properties (e.g., pH levels) (Diogo et al., 2024; Liu et al., 2014; Marinho et al., 2019; Tian et al., 2024). AChE activity is essential for neurotransmission, breaking down acetylcholine to regulate nerve signals (Ellman et al., 1961). The disruption of AChE activity can cause acetylcholine accumulation, leading to negative effects, such as behavioral alterations, developmental issues, and weakened defense mechanisms (Huo et al., 2023). Similarly to SMX, under acidic pH, the activity of AChE decreased significantly after TRIM exposure (Figs. 1 and 6), likely due to protonation effects that disrupt enzyme-substrate

interaction, as previously discussed (*see* previous section). At higher pH levels (7.5 and 9.0) an increase in AChE activity was noted after exposure to TRIM (Figs. 2, 3 and 6), which corroborates with previous studies performed in *D. rerio* following exposure to sulfonamides and TRIM, attributing the alterations caused in AChE activity to the mechanism of action of these antibiotics (Crivello et al., 2010; Diogo et al., 2024, 2025b; Huo et al., 2023; Lee et al., 2012).

4.3. Toxicity of mixture under pH variations

The interaction analysis using the IA model revealed that the combined effects of SMX and TRIM are not merely additive (Figs. 1, 2, and 3). Instead, a complex interplay of antagonistic and synergistic responses was observed, varying according to the biological parameter and pH scenario (Figs. 1 to 4II). These interactions suggest that one compound may modulate or counteract the effects of the other, leading to unexpected outcomes that cannot be predicted from individual exposures alone. At acidic pH (6.5; Fig. 1), the interaction between SMX and TRIM showed a balance of antagonistic and synergistic effects across biomarkers. Some oxidative stress and metabolic parameters decreased more than expected (antagonism), while others related to oxidative damage and energy metabolism were enhanced (synergism), indicating complex and, non-additive responses. At neutral pH (7.5; Fig. 2), antagonistic down interactions were dominant across most endpoints, indicating that the combined effect of SMX and TRIM was lower than expected based on their individual actions. Under alkaline conditions (pH 9.0; Fig. 3), additive effects were observed for SOD activity and lipid content, indicating that the combined effects of SMX and TRIM on these parameters were consistent with the sum of their individual actions. In contrast, a synergistic suppression of energy-related parameters, alongside antagonistic down interactions in oxidative stress and genotoxicity markers, revealed combined effects that were lower than anticipated from individual exposures. Altogether, these outcomes highlight the critical role of environmental context, particularly pH, in shaping mixture toxicity, as chemical interactions can amplify or dampen biological responses in a parameter-specific and non-linear manner.

Thus, the results indicate that the mixture of SMX and TRIM significantly disrupts antioxidant and detoxification mechanisms (e.g., CAT, GRed, and GSTs activities), leading to lipid peroxidation and DNA damage, across all the pH values tested (Fig. 7). Both acidic and alkaline conditions can induce pH stress (Fig. 7), stimulating intracellular ROS production and impacting antioxidant defenses, immune responses, and physiological indicators such as hematological parameters (Kim et al., 2021; Qu et al., 2014). Although no specific data exists on toxicity of SMX and TRIM mixture across pH variations, several studies report the influence of pH on the toxicity of pharmaceutical mixtures. For example, Liu et al. (2014) found that SMX combined with norfloxacin at pH 7.0 increased DNA damage in the gonads of *Carassius auratus* after a 7-day exposure. Li et al. (2012) observed increased GST activity in *C. auratus* exposed to SMX concentrations above 16 μg/L and SMX-caffeine mixtures under neutral pH. Similarly, Yang et al. (2019) showed that a mixture of SMX, ofloxacin, and ibuprofen caused significant alterations in SOD activity, while Ramesh et al. (2018) found that antioxidant defenses (SOD, CAT, and GPx) in *Cirrhinus mrigala* were disrupted by sulfamethazine exposure at neutral pH. Yan et al. (2016) reported similar oxidative responses in *D. rerio* exposed to a SMX-norfloxacin mixture. Zhou et al. (2009) suggest that cytoplasmic pH changes in aquatic animals due to environmental pH modification can act as a critical signal for cytokine and chemokine synthesis and release, subsequently promoting ROS generation. Qu et al. (2014) further indicate that oxidative effects induced by pH may vary across different tissues based on their sensitivity to oxidative stress. Certain tissues (e.g., gills and liver) are more vulnerable to oxidative damage due to their direct exposure to environmental stressors and their crucial roles in detoxification and metabolism. These tissue-specific variations highlight the complexity of oxidative stress responses under different pH conditions and underscore the need to consider multiple biomarkers when assessing environmental toxicity. The physiological disruptions weaken the organism's ability to withstand additional stressors, reducing overall fitness and potentially affecting population dynamics (Moiseenko, 2005). Over time, such changes can ripple through the ecosystem, decreasing biodiversity, altering species distribution, and impacting key ecological processes critical to ecosystem stability (Cornwall et al., 2023).

Different authors reported that pH can affect the energetic metabolism of different organisms exposed to pharmaceuticals (e.g., Almeida

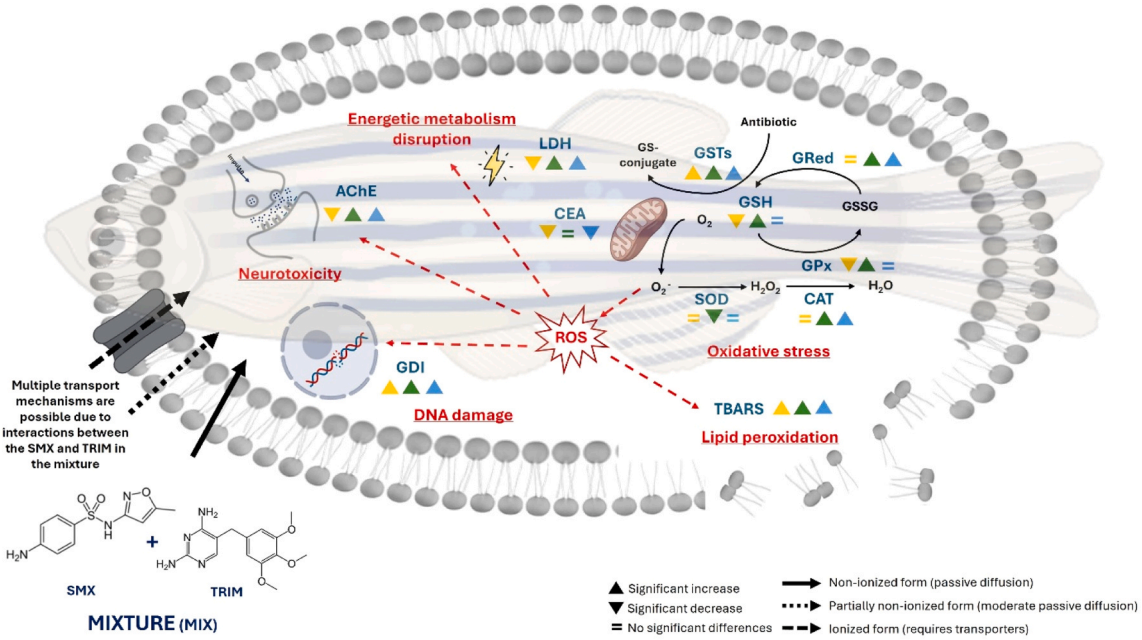

Fig. 7. Description of the mechanisms of toxicity for antibiotic mixture (MIX) across different pH conditions (6.5, 7.5, and 9.0), according to the results of biochemical responses illustrating how pH influences MIX toxicity. Key pathways and effects are color-coded by pH condition: yellow for pH 6.5, green for pH 7.5, and blue for pH 9.0, highlighting variations in toxicity mechanisms across the tested pH range.

et al., 2022; Costa et al., 2020; Yildiz and Altunay, 2011). Exposure of *D. rerio* to MIX resulted in the same trend observed with SMX, with metabolic alterations indicating an imbalance between energy demand and availability (Figs. 5 and 7). The reduction in CEA and decreased LDH activity at acidic pH suggests a lower reliance on anaerobic pathways (Fig. 1). This could be due to an increased dependence on aerobic metabolism, as oxidative stress and cellular damage at low pH may require more efficient ATP production through mitochondrial respiration. The reduced LDH activity implies a decreased conversion of pyruvate to lactate, a key step in anaerobic glycolysis, potentially reflecting an adaptive response to maintain cellular homeostasis under acidic conditions. However, this shift may also indicate an energy imbalance, where the organism struggles to meet its energetic demands, ultimately affecting physiological functions and overall fitness. Similar metabolic adjustments have been reported in other aquatic organisms, where exposure to acidic pH levels led to a shift toward anaerobic metabolism. This response is often characterized by increased LDH activity and a reduction in key aerobic enzymes, suggesting a compensatory mechanism to maintain energy production under environmental stress (e.g., Pimentel et al., 2019; Cao et al., 2018 – *see section* 4.1). At neutral pH, the increase in LDH indicates a possible shift toward anaerobic metabolism (Fig. 2), reinforcing the combined impact of the MIX and pH variations on the organism's energy homeostasis (Figs. 2 and 7). Yildiz and Altunay (2011) reported that the SMX and TRIM mixture (40 μg/L; ratio 5:1) triggers a metabolic stress response in *Dicentrarchus labrax* and *Sparus aurata* at a pH of 7.5. These authors observed that levels of cortisol, glucose, and plasma ions increased in these species, functioning as adaptive mechanisms to maintain homeostasis. This response resulted in the hypersecretion of catecholamines and corticosteroids, which subsequently led to elevated blood glucose levels in both species (Yildiz and Altunay, 2011). This rise in glucose, driven by glycogenolysis and sustained by gluconeogenesis, is a typical reaction to metabolic stress. Enhanced glucose production is an adaptation that helps tissues meet the higher energy demands associated with stressful conditions (Yildiz and Altunay, 2011). Under alkaline pH, a similar pattern has been observed in other species, where decreased energy reserves and increased Na^+/K^+-ATPase activity suggest a metabolic shift prioritizing ion regulation over other physiological processes (Bolner et al., 2014).

Changes in pH can alter the enzyme's structure and function, including AChE, thereby affecting its activity (Marinho et al., 2019). Such pH fluctuations can disrupt neuronal excitability, synaptic communication, neurotransmitter transport, and intercellular signaling, emphasizing the critical importance of pH homeostasis in maintaining nervous system function and overall organismal health (Serova et al., 2020). A significant decrease in AChE activity was observed under acidic pH, after MIX exposure, which aligns with the results obtained in SMX and TRIM individually (Figs. 1 and 7). Diogo et al. (2025b) provided a comprehensive summary of the mechanisms affected by SMX and TRIM exposure in aquatic species, highlighting that species, concentrations, and exposure times can influence the response of AChE activity, shaping how this enzyme is affected across different exposure conditions. As mentioned before (*see sections* 4.1 and 4.2), at acidic pH, the protonation of histidine residues within the active site of AChE is thought to interfere with its substrate binding, reducing enzyme activity (Marinho et al., 2019). This mechanism was observed in *D. rerio* following exposure to both individual antibiotics (SMX and TRIM) and their mixture (Fig. 1). Similar pH-induced changes in AChE activity were observed, indicating that altered pH conditions similarly disrupt enzyme function. Following the pattern of TRIM exposure, at higher pH levels (7.5 and 9.0), a significant increase in AChE activity was observed after exposure to MIX (Figs. 2 and 3). Such increases in AChE activity may represent a compensatory mechanism in response to induced neurotoxicity, reflecting an adaptive attempt by *D. rerio* to maintain neurophysiological function. Similar findings have been reported by other studies, highlighting that environmental changes, including pH shifts, can exacerbate the neurotoxic effects of antibiotics to aquatic species, further threatening their survival and overall health (Turhan, 2021). Specifically, other authors have shown that the AChE activity of zebrafish increased significantly after exposure to environmentally relevant concentrations of antibiotics (e.g., ciprofloxacin, enrofloxacin) and elevated pH levels (> 7.0), suggesting that both factors contribute to neurotoxic stress in these species (e.g., Turhan, 2021).

5. Ecotoxicity assessment and biological health status in *Danio rerio*: The combined effects of pH and antibiotics

The simultaneous occurrence of pH fluctuations and antibiotic contamination is particularly concerning, as these factors may interact and amplify their harmful effects on the aquatic environment (Bethke et al., 2023). The results of this study demonstrate that environmentally relevant concentrations of SMX, TRIM, and MIX, combined with pH fluctuations, significantly impact the health and well-being of juvenile *D. rerio* (Figs. 5, 6 and 7). These findings indicate that under fluctuating environmental conditions, antibiotic combinations can severely compromise aquatic organisms' health, leading to increased oxidative stress and cellular damage that disrupt essential functions, such as growth and reproduction.

SMX was revealed to be marginally toxic to *D. rerio* in all tested pH conditions (Table 3). However, its impact on health status varied with pH, causing more severe alterations under acidic conditions, while leading to major and moderate alterations at neutral and alkaline pH, respectively (Table 3). At acidic pH, as a weak acid, SMX remains in its non-ionized (neutral) form, enhancing its ability to cross cell membranes (Fig. 5), leading to greater absorption and toxicity in *D. rerio* (Montone et al., 2024), as evidenced by severe alterations observed (e.g., oxidative stress and DNA damage; Figs. 1 and 4). In contrast, the toxicity of TRIM increased with alkaline pH (Table 3). This antibiotic is slightly toxic at acidic pH and causes major alterations (e.g., oxidative stress; Fig. 1) in *D. rerio* (Table 3). However, at neutral and alkaline pH, toxicity increases, leading to more severe alterations (e.g., lipid peroxidation, neurotoxicity; Figs. 2 and 3 and Table 3). As a weak base, TRIM remains in a non-ionized form, which enhances its permeability across cell membranes (Fig. 6), resulting in increased absorption and toxicity for *D. rerio* (AlRabiah et al., 2018). Regarding MIX, the results suggested that toxicity also varies with pH (Fig. 7), causing negative effects on *D. rerio* health (e.g., oxidative stress, lipid peroxidation, DNA damage, and neurotoxicity; Figs. 1 and 2). MIX was marginally toxic at acidic and alkaline pH conditions and moderately toxic at neutral pH (Table 3). At acidic pH, only major alterations, such as oxidative stress and lipid peroxidation, were observed (Fig. 1). In contrast, more severe alterations occurred at neutral and alkaline pH (Figs. 2 and 3). This increased toxicity suggests that the combination of SMX and TRIM becomes more harmful under these conditions, possibly due to greater absorption or cellular reactivity at these pH levels.

6. Conclusions and future perspectives

This study demonstrates that the toxicity of the antibiotics, both individually and in mixtures, is significantly influenced by pH fluctuations. These variations create distinct toxicity profiles that can compromise aquatic organisms' health and threaten ecosystem stability (Almeida et al., 2022; Zhang et al., 2023), ultimately amplifying the risks of biological and ecological disruption (Alsop and Wilson, 2019; Bethke et al., 2023). The observed physiological and metabolic disturbances highlight how antibiotic mixtures, under varying pH conditions, can escalate oxidative stress and cellular damage, impairing critical functions such as immune response, locomotion, and reproduction. The cumulative impact of these disruptions, intensified by pH variations, weakens individual resilience, alters population dynamics, and destabilizes aquatic ecosystems by affecting biodiversity and trophic interactions (Alsop and Wilson, 2019; Cornwall et al., 2023). These

findings emphasize the urgent need to study the combined effects of multiple environmental pressures, including chemical contamination and abiotic stressors driven by climate change (e.g., temperature fluctuations and pH shifts), as they often co-occur in natural ecosystems. Understanding how these stressors interact is essential for assessing their risks to biodiversity and ecosystem functioning, highlighting the importance of integrative approaches in environmental risk assessments.

Future research should expand this knowledge by exploring long-term exposures, different life stages, and multi- and transgenerational effects of contaminants under environmentally relevant scenarios. Additionally, it is crucial to investigate mixture toxicity involving other classes of pharmaceuticals and emerging contaminants under variable environmental conditions. Such insights are essential to inform environmental policies better, improve ecological risk assessment frameworks, and guide water management strategies in a changing climate.

CRediT authorship contribution statement

Bárbara S. Diogo: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Oksana Golovko:** Writing – review & editing, Methodology. **Sara Rodrigues:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **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

Not applicable.

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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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2025.104774.

Data availability

All data are present in the manuscript

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