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Influence of sucralose, acesulfame-k, and their mixture on brain's fish: A study of behavior, oxidative damage, and acetylcholinesterase activity in *Danio rerio*

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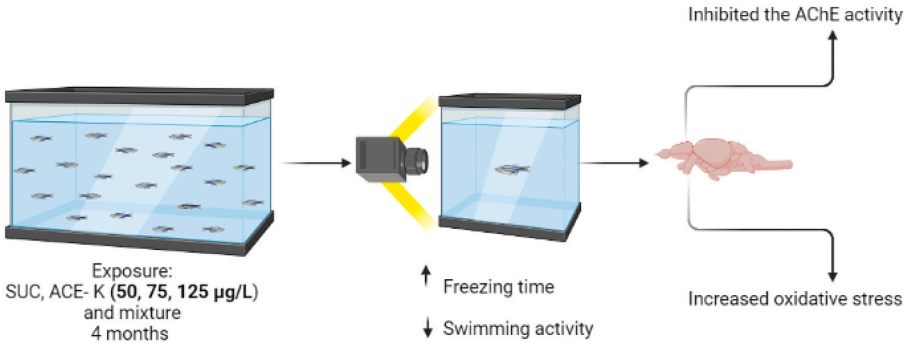
HIGHLIGHTS

- Sweeteners significantly increase the production of free radicals in the brain.
- An anxiety behavior was observed in fish exposed to both sweeteners.
- Acesulfame K generated a more harmful response than sucralose.
- Chronic exposure to sweeteners decreased acetylcholinesterase activity in fish.

GRAPHICAL ABSTRACT



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ABSTRACT

Sucralose (SUC) and acesulfame-k (ACE-K) are widely used artificial sweeteners worldwide; however, they are frequently detected in aquatic environments due to their low metabolism and inadequate removal during wastewater treatment. The harmful effects of these compounds on hydrobionts have yet to be fully understood, as data on their toxicity is limited and inconclusive. This research aimed to determine the impact of SUC (50, 75, 125 µg/L) and ACE-K (50, 75, 125 µg/L), individually and in combination, on fish's swimming behavior, acetylcholinesterase activity, and oxidative stress response after four months of exposure. Following exposure, adult *Danio rerio* displayed anxiety-like behavior, as evidenced by increased freezing time and decreased swimming activity. Additionally, analysis of fish brain tissue revealed a disruption of REDOX homeostasis, leading to oxidative stress, which may be responsible for the observed inhibition of AChE activity. The results indicated that ACE-K was more toxic than SUC, and the mixture of both compounds produced a more detrimental effect than when each compound was administered alone. These findings highlight the hazardous impacts of SUC

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and ACE-K on fish in environmentally relevant concentrations, suggesting that these compounds should be added to the priority pollutant list.

1. Introduction

Due to their low or negligible caloric content, non-nutritive sweeteners have become popular among individuals seeking to maintain a healthy lifestyle and prevent conditions such as acne, diabetes, and obesity (Dietrich et al., 2021). The global consumption of artificial sweeteners currently stands at over 1.6×10^3 metric tons, with a market value of US$ 2 billion (Praveena et al., 2019). However, recent projections suggest that the global market for these sweeteners will surge to US$ 9.7 billion by 2024 (PRNewswire, 2019). China, with a consumption rate of 32%, is the largest consumer of non-nutritive sweeteners, followed by Oceania-Asia (23%), the United States (23%), Europe (12%), and Africa (7%). According to Euromonitor International (2017), aspartame is the most widely used non-nutritive sweetener in the food and beverage industry, with 18.5×10^3 metric tons, followed by saccharine (9.7×10^3 metric tons), acesulfame (6.8×10^3 metric tons), and sucralose (3.3×10^3 metric tons). In recent times, a great deal of attention has been devoted by the scientific community to acesulfame (ACE-K) and sucralose (SUC) due to their inadequate mammalian metabolism and limited removal by wastewater treatment plants (WWTPs). As per the findings of Dietrich et al. (2021), certain sweeteners such as aspartame, cyclamate, neotame, and saccharin can be effectively biodegraded in WWTPs. However, ACE-K and SUC exhibit poor to negligible biodegradability. Furthermore, traditional treatment techniques such as advanced oxidation activated carbon and chlorination have proven ineffective in breaking down both sweeteners in

Table 1
Worldwide occurrence of ACE-K and SUC in aquatic environments.

Continent	Country	Aquatic matrix	Concentration (μg/L)	References
Sucralose				
Asia	China	Ground water	0.0129	Gan et al. (2013a)
		Tap water	0.113–0.171	Gan et al. (2013a)
		Surface water	0.287–0.311	Gan et al. (2013b)
		Surface water	0.05	Sang et al. (2014)
		Surface water	0.89–20.6	Fu et al. (2020)
	Singapore	Ground water	<0.03	Tran et al. (2013)
		Surface water	>0.05	Tran et al. (2013)
Europe	Finland	Surface water	<0.2–1	Perkola and Sainio (2014)
	Spain	Effluent wastewater	0.5	Ordóñez et al. (2012)
		Surface water	25	Ordóñez et al. (2012)
		Effluent wastewater	0.05–155	Arbeláez et al. (2015)
		Surface water	3.57	Arbeláez et al. (2015)
	Sweden	Surface water	0.004	Gan et al. (2013a)
	Switzerland	Surface water	<0.01–0.949	Berset and Ochsenbein (2012)
North America	EE. UU.	Effluent wastewater	27	Oppenheimer et al. (2011)
		Surface water	0.12–10	Oppenheimer et al. (2011)
		Effluent wastewater	77	Cantwell et al. (2019)
		Surface water	0.018–3.18	Cantwell et al. (2019)
		Influent wastewater	0.9176–2.0312	Yang et al. (2021)
		Surface water	2.07	Yang et al. (2021)
		Drinking water	0.2881–0.5053	Yang et al. (2021)
Acesulfame				
Africa	Nigeria	Effluent wastewater	16	Hu et al. (2021)
Asia	China	Effluent wastewater	46	Gan et al. (2013)
		Surface water	0.013–0.32	Wang et al. (2019)
		Drinking water	0.093–0.125	Zhou et al. (2021)
		Surface water	0.40–2.78	Fu et al. (2020)
	Korea	Ground water	0.0329	Lee et al. (2019)
	Singapore	Effluent wastewater	4.33–135.76	Tran et al. (2014)
		Effluent wastewater	6.316–10.513	Tran et al. (2015)
Europe	Czech Republic	Influent wastewater	22.67	Kerberová et al. (2021)
	Germany	Ground water	34	Lange et al. (2012)
		Surface water	21	Scheurer et al. (2011)
		Drinking water	7	Lange et al. (2012)
	Italy	Effluent wastewater	2500	Loos et al. (2013)
	Norway	Surface water	25	Kokotou et al. (2012)
	Serbia	Ground water	0.02–0.017	Gvozdić et al. (2020)
	Spain	Effluent wastewater	155	Arbeláez et al. (2015)
		Effluent wastewater	0.05	Arbeláez et al. (2015)
		Surface water	1.62	Arbeláez et al. (2015)
	Switzerland	Ground water	0.524	Ens et al., 2014
		Effluent wastewater	12–46	Buerge et al. (2011)
		Ground water	4.7	Buerge and Poiger, 2011
		Tap water	2.6	Buerge and Poiger, 2011
		Ground water	5	Buerge, 2011
North America	Canada	Ground water	0.541	Wu et al., 2014
		Surface water	0.094	Sérodes et al. (2021)
	USA	Effluent wastewater	10–50	Oppenheimer et al., 2011
Oceania	Australia	Irrigation water	0.35–0.61	Mora et al., 2021
		Ground water	0.005–0.34	Mora et al., 2021
South America	Brazil	Effluent wastewater	13–31	Alves et al., 2021

wastewater (Buerge et al., 2009; Scheurer et al., 2010; Li et al., 2018; Jahani et al., 2020). Consequently, the extensive usage and inadequate removal of these sweeteners have led to their global distribution and occurrence in aquatic environments (as indicated in Table 1).

Once introduced into the environment, sweeteners can induce various toxic responses in aquatic organisms, disrupting their homeostasis. For instance, Cruz-Rojas et al. (2019) demonstrated that ACE-K at concentrations of 0.05 and 149 μg/L significantly induced oxidative stress in the gill, brain, muscle, liver, and blood of *Cyprinus carpio*. Concerning SUC toxicity, Eriksson Wiklund et al. (2014) found that concentrations ranging from 0.001 to 5 mg/L triggered oxidative damage and increased acetylcholinesterase activity in *Daphnia magna*. Likewise, Saucedo-Vence et al. (2017) observed that SUC at 0.05 and 155 μg/L induced oxidative stress in the liver, gill, brain, and muscles of *Cyprinus carpio*. Furthermore, Saputra et al. (2021) and Colín-García et al. (2022) demonstrated that SUC (0.05–116.5 μg/L) impaired the normal development of *Danio rerio* embryos, resulting in various malformations and increased heart rate.

Overall, the scientific community has not raised concern about the neurotoxic effects prompted by SUC and ACE-K. To our knowledge, only one study has reported an adverse impact of ACE-K on the brain of adult *Danio rerio*, wherein the authors found that ACE-K disrupted behavior and altered the quantitative patterns of neurotransmitters in the brain (Dong et al., 2020). However, the concentrations of ACE-K used in this study (1–100 mg/L) were significantly higher than those in the natural aquatic environment. Given this information and the absence of previous research examining the detrimental effects that the mixture of sweeteners may pose to the brain's fish, this study aimed to investigate whether realistic concentrations of SUC and ACE-K, either alone or in combination, would negatively impact the behavior of *Danio rerio* adults. Additionally, this research evaluated whether such concentrations would disrupt zebrafish's redox status and acetylcholinesterase activity. As sweeteners can induce oxidative stress in fish and H_2O_2, a by-product of reactive oxygen species, is known to inhibit acetylcholinesterase activity (Elizalde-Velázquez et al.,2022a, 2022b), it is hypothesized that the exposure to said compounds, whether singularly or in conjunction, shall give rise to aberrant behavior in fish.

2. Method

2.1. Ethics committee approval

This research protocol underwent a thorough review and obtained approval from the Ethics and Research Committee of the Autonomous University of the State of Mexico (UAEM). The approval ID for this protocol is RP. UAEM. ERC.150.2022. Furthermore, the provisions outlined in the Mexican official standard on the breeding, care, and use of laboratory animals (NOM-062-ZOO-1999) were duly considered and adhered to throughout the research.

2.2. Compounds

ACE-K (CAS: 55 589–62-3), SUC (CAS: 56 038-13-2), and the remaining compounds were purchased from Sigma Aldrich (St. Louis, MO).

2.3. Fish upkeep

Female and male *Danio rerio* adults (5 month-old; AB strain) remained housed, in a ratio of 1 fish/L, in aquaria of 100 L capacity. Every aquarium was equipped with an open water system that received a continuous supply of aerated, dechlorinated, charcoal-filtered, and UV-sterilized tap water. All organisms used for this research were born and grown up in the laboratory. Table 2 shows the water quality parameters measured every other day of all aquaria during fish maintenance and exposure. Temperature (27 ± 1 °C) and dark/light cycles (14:10 h) were upheld consistently across all aquaria to ensure organisms were kept healthy. Feeding was performed two times a day with Spirulina flakes (Ocean Nutrition, US) and living *Artemia nauplii*.

Table 2
Quality of water during upkeep and exposure of fish.

Parameters	Measured value
O_2	9.2 ± 0.2 mg/L
NO_2^-	0.025 ± 0.008 mg/L
$NO3^-$	2.8 ± 0.4 mg/L
pH	7.33 ± 0.18
NH_3	0.010 ± 0.003 mg/L

Values are expressed as mean ± S.D.

2.4. Exposure

A total of 640 fish were assigned to 16 systems, each containing 40 housed in 40 L tanks. Each system was allocated to one of the tested concentrations, including a control group, as outlined in Table 3. The concentration range selected for this experiment was consistent with those previously reported in surface waters, including those known to have toxic effects on aquatic species (please refer to Table 1). Over four months, water in all systems was renewed every other day, while temperature (27 ± 1 °C) and light/dark cycles (14 h:10 h) were maintained constant throughout the exposure period. The same exposure procedure was repeated thrice with different organisms, as three independent experiments were performed for this research.

2.5. Behavioral assessment

After four months of exposure, the treatments, but the control group, displayed a mean survival rate of 85% fish (with a standard deviation of ±5%). The control group exhibited a mean survival rate of 92.5%, with a standard deviation of ±2.5%. It should be noted, however, that only a randomly selected subset of 30 fish from each treatment group was used for the behavioral evaluation. Herein, the fish's behavior was assessed through two tests: 1) NovelTank and 2) Dark/Light. For each of these, half of the selected organisms were used. Thus, 15 fish were used per test. No fish were utilized twice or were subjected to a repeated stimulus. The exact above-described procedure was repeated with organisms from the three independent experiments. The mean of three independent results was calculated to be depicted in bar charts.

2.5.1. NovelTank test

To conduct the NovelTank Test, the procedure described by Elizalde-Velázquez et al. (2022a) was followed. All fish were transferred from the exposure room to the behavioral room and acclimatized for 50 min. The behavioral room was soundproof and did not allow light to

Table 3
Concentrations of SUC, ACE-K, and their mixtures evaluated herein.

Treatments	Concentrations (μg/L)
SUC 1	50
SUC 2	75
SUC 3	125
ACE-K 1	50
ACE-K 2	75
ACE-K 3	125
M1 (SUC + ACE-K)	50 + 50
M2 (SUC + ACE-K)	50 + 75
M3 (SUC + ACE-K)	50 + 125
M4 (SUC + ACE-K)	75 + 50
M5 (SUC + ACE-K)	75 + 75
M6 (SUC + ACE-K)	75 + 125
M7 (SUC + ACE-K)	125 + 50
M8 (SUC + ACE-K)	125 + 75
M9 (SUC + ACE-K)	125 + 125

pass, but it maintained the same temperature as the exposure room. Following acclimatization, one fish at a time was placed into a 15 L rectangular novel tank (21.2 cm × 21.2 cm × 25.2 cm) for 12 min, with 2 min for acclimatization and 10 min for the trial. None of the tanks contained SUC, ACE-K, or their mixtures, and the water in all tanks fulfilled the parameters established in Table 2. During the 10-min trial, a video recording was made, which was subsequently analyzed using Ethovision XT 13.0 software. The endpoints measured were the distance traveled in the top and bottom (cm), time spent in the top and bottom (s), total distance traveled (cm), and time fish remained frozen in the top and bottom (s). The NovelTank Test was performed on different days and only in the morning (8 a.m.–10 a.m.) to avoid any behavioral disturbance during the experiment.

2.5.2. Dark/light experiment

Following the procedures outlined in the NovelTank Test, the fish were relocated from the exposure area to the behavioral room and remained there for 50 min. Moreover, the water from all tanks fulfilled the parameters established in Table 2 and was free of SUC and ACE-K. This experiment required acrylic tanks with dimensions of 15 cm × 10 cm x 45 cm and divided into two equal parts, half of the tank black and the other half white. The material used on the black side of the tank was anti-reflective to avoid any fish behavioral disturbance during the test. To conduct the test, a single fish was placed in the tank for 12 min, consisting of 2 min of acclimatization followed by 10 min of the trial. The footage was captured during the trial period. The time spent in the black-and-white area (s) and the number of freezing bouts were the endpoints considered for this behavioral assessment. The videos were analyzed using Ethovision XT 13.0 software. The Dark/Light Test was conducted on different days, exclusively during the morning hours of 8 a.m.–10 a.m., to preclude any potential disruptions to the subjects' behavior during the investigation.

2.6. Brain gathering and AChE determination

After assessing behavior, the hypothermic shock method (Underwood and Anthony, 2020) was utilized to euthanize the fish. Accordingly, a mean of 34 brains (with a standard deviation of ±2) were extracted per treatment group. Extracted brains were allocated in Eppendorf tubes with 1 mL of PBS (pH 7.4). Three Eppendorf tubes were obtained for all treatments, including the control group, each containing a mean of 11 brains (with a standard deviation of ±1). Two tubes were used to assess each of the following biomarkers: oxidative stress and acetylcholinesterase activity. Moreover, the third tube was used to quantify the amount of SUC and ACE-K in the brain. Fish used in the three independent experiments underwent the same euthanasia and dissection procedure after the behavioral test. Accordingly, 11-brain pools from the three independent experiments were used to evaluate AChE activity, oxidative damage, and the quantification of chemicals in the brain. Additionally, each sample underwent evaluation three times for all biomarkers. Therefore, nine results were obtained per biomarker and treatment group (n = 9). To evaluate AChE activity, the samples were treated as Elizalde-Velázquez et al. (2022b) described. Specifically, brains were homogenized using a rotor-stator homogenizer (Ultra--turrax T25, IKA, Germany) for 30s. The resulting homogenized was then centrifuged at 10 000 rpm for 15 min at 4 °C, and 400 μg/L of the supernatant was mixed with 2.6 mL of phosphate buffer (pH 8.0, 0.1 M), 0.1 mL of 5,5-dithiobis-2-nitrobenzoate (0.1 M), and 25 μL of the substrate (ACh iodide, 0,075 M). Changes in absorbance at a wavelength of 412 nm were recorded over 5 min.

2.7. Oxidative stress evaluation

Samples were subjected to the protocol described by Elizalde-Velázquez et al. (2021a),b. Specifically, the homogenate was divided into two Eppendorf tubes. Tube 1 contained 300 μ L of homogenate and 300 μ L of 20% TCA (trichloroacetic acid), while tube 2 contained 700 μ L of homogenate. Subsequently, centrifugation was performed on both tubes, and the resulting supernatant and precipitate were used to evaluate various biomarkers, including antioxidant enzymes (superoxide dismutase and catalase), lipoperoxidation, hydroperoxides, and protein carbonyl content. The results were normalized against total proteins, and the methods for each technique are described in detail in Table 1S.

2.8. SUC and ACE-K quantification

SUC and ACE-K were quantified in the brain and all water samples. A 10 mL water sample from each system was collected every week before the renewal of the medium. All water samples were stored at −20 °C until their quantification. To quantify SUC and ACE-K in the brain, the homogenized was mixed with 4 mL of methanol: water (1:1) and centrifuged at 2500×*g* for 5 min. The top layer was then subjected to further analysis using HPLC-MS/MS. Compound determination was performed using an Agilent 1290 Infinity HPLC unit and an Agilent 6430 Triple Quadruple MS equipped with electrospray. In both cases, separation was achieved through an RRHD Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm). To quantify ACE-K, the mobile phase comprised ultrapure water and acetonitrile containing ammonium acetate (5 mM) and TRIS (1 mM). Meanwhile, the mobile phase consisted of water with formic acid (pH 2.5) and acetonitrile for SUC quantification. The flow rate and injection volume were set at 0.4 mL/min and 30 μL, respectively. Negative ESI was optimized for the analysis of both compounds under the following conditions: 45 psi (nebulizer pressure), 11 L/min N2 (drying gas flow), 350 °C (drying gas temperature), and 4000 V (capillary voltage).

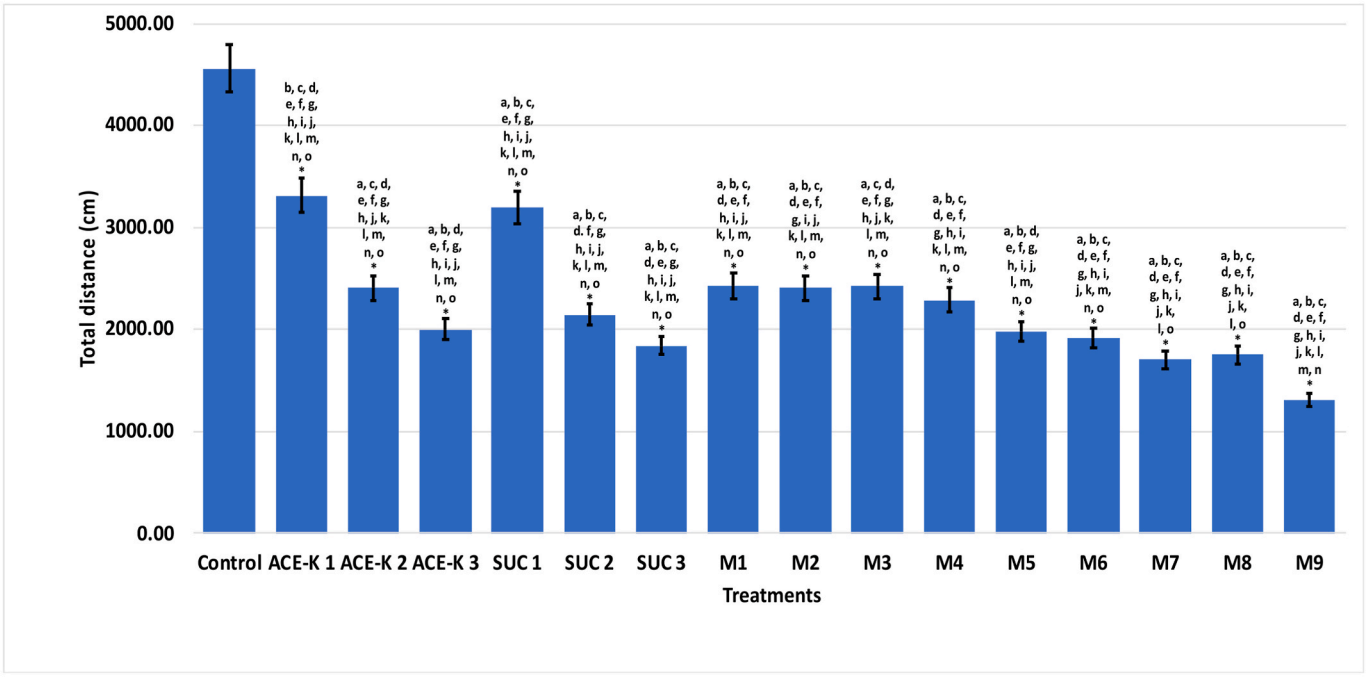
2.9. Statistical analysis

The homoscedasticity and normality of the data were assessed using the Bartlett test for homogeneity of variances and the Shapiro-Wilk test for normality, respectively. Significant differences in means between treatment groups were determined using a Student Newman Keuls test with a significance level of $p < 0.05$ and a one-way ANOVA with a significance level of $\alpha = 0.05$. The results were reported as mean ± standard deviation (SD) and analyzed using SigmaPlot 12.3 software. A Pearson correlation analysis was performed to assess the strength of the association between oxidative stress biomarkers and behavioral test endpoints, as well as the relationship between these variables and acetylcholinesterase activity. The significance level was set at $p < 0.05$, and the analysis was conducted using R software. The resulting correlations were visualized using a heatmap. The results for response charts and correlograms were normalized to obtain the relative response of biomarkers based on control values.

3. Results

3.1. Novel tank test

All endpoints assessed during the test (Fig. 1A–D) were found to be altered by individual artificial sweeteners and their mixtures. For instance, a significant decline in the total distance traveled was observed in fish exposed to both compounds and their combinations compared to the control group. The effects were more severe in the mixtures than in the compounds alone. Although a significant reduction in the distance traveled at the bottom was not observed in fish exposed to SUC and ACE-K, our results indicated a considerable decrease in the length traveled at the top in all treatment groups compared to the control group. This is



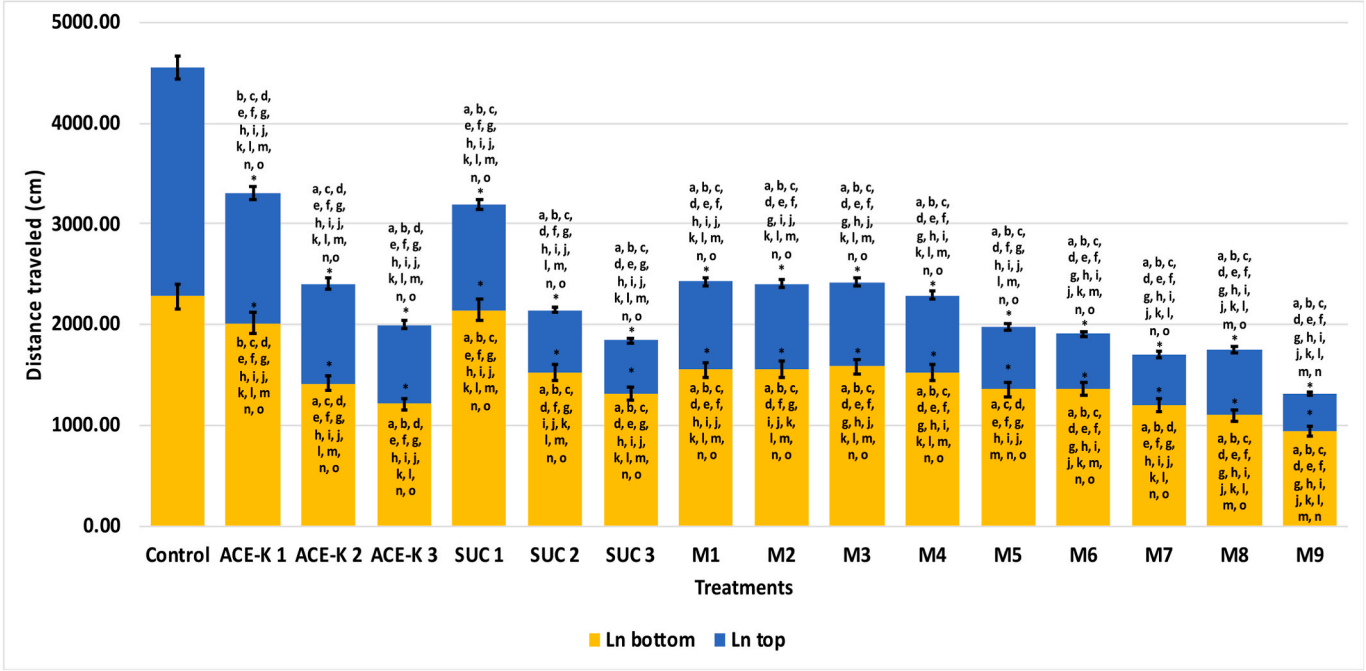
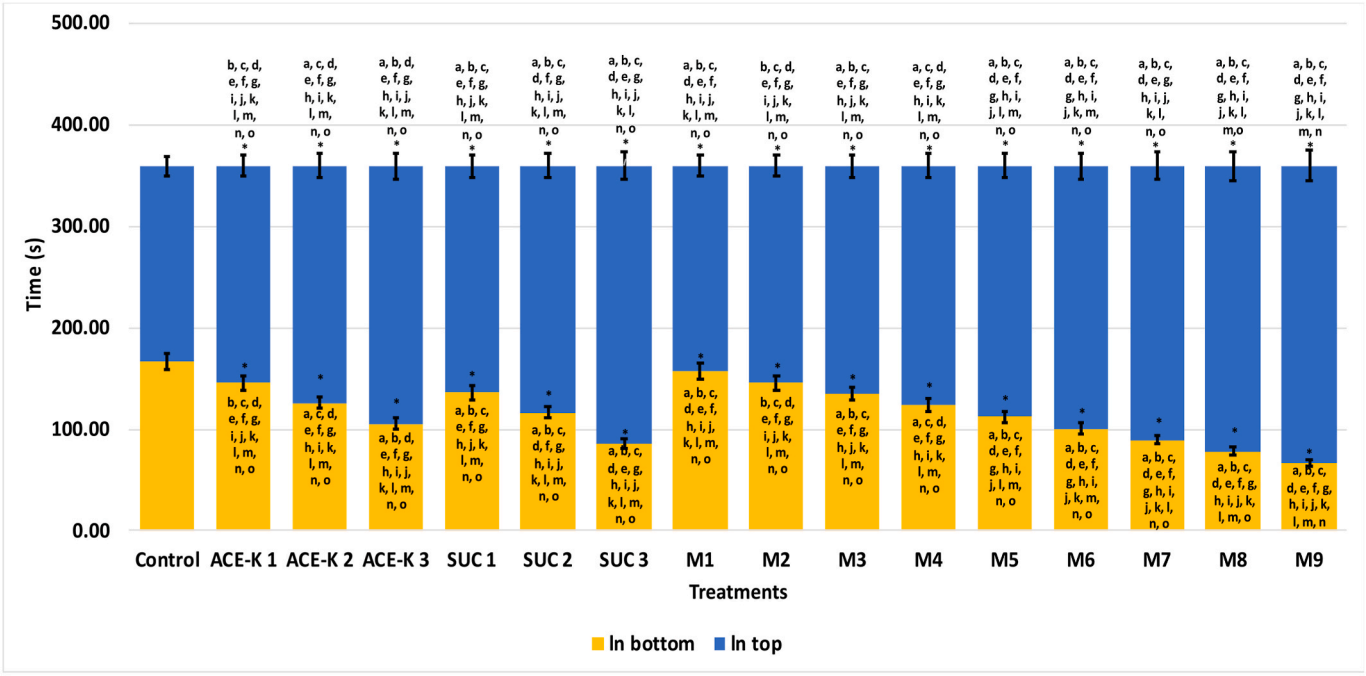


Fig. 1. NovelTank Test results (A: Total distance traveled, B: Distance traveled in top and bottom, C: Time spent in top and bottom, D: Freezing time) of fish exposed to SUC, ACE-K, and their mixtures. Data represent the mean ± S.D. of three independent experiments, n = 45. * indicate a significant change compared to the control group. A: indicate a significant change compared ACE-K1. B: indicate a significant change compared ACE-K2. C: indicate a significant change compared ACE-K3. D: indicate a significant change compared SUC1. E: indicate a significant change compared SUC2. F: indicate a significant change compared SUC3. G: indicate a significant change compared M1. H: indicate a significant change compared M2. I: indicate a significant change compared M3. J: indicate a significant change compared M4. K: indicate a significant change compared M5. L: indicate a significant change compared M6. M: indicate a significant change compared M7. N: indicate a significant change compared M8. O: indicate a significant change compared M9.

C



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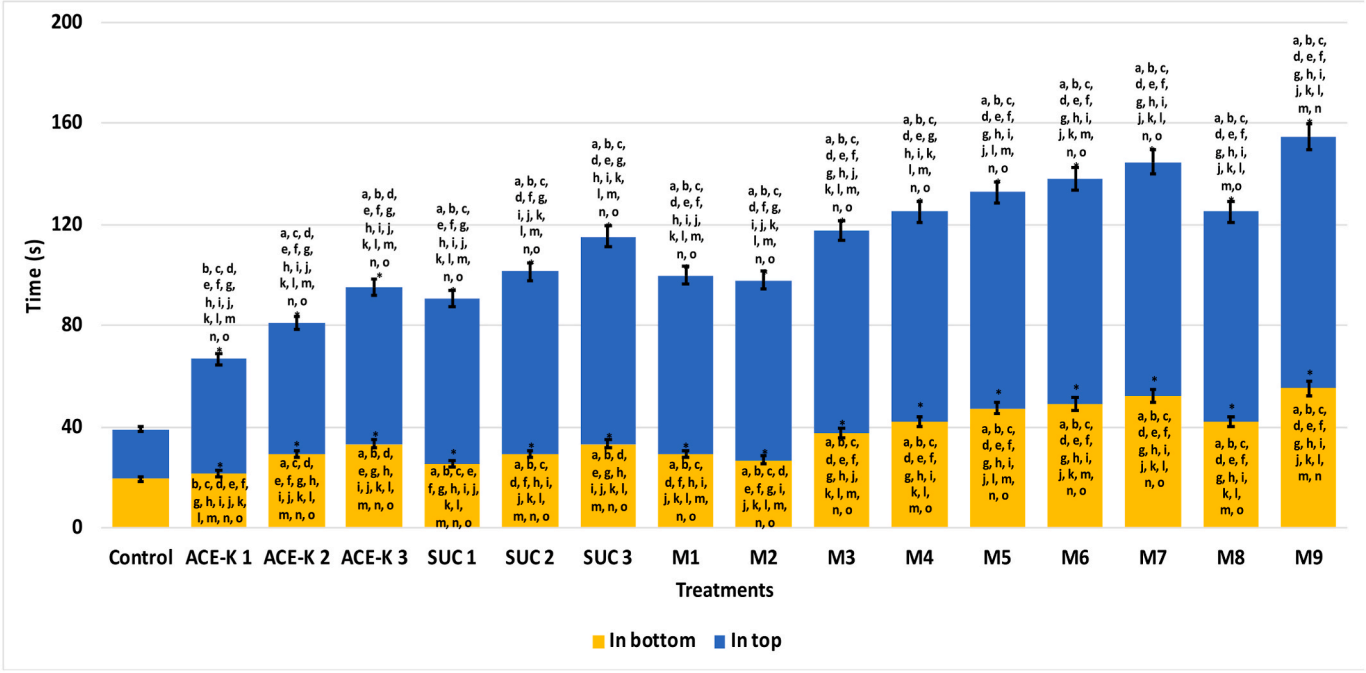
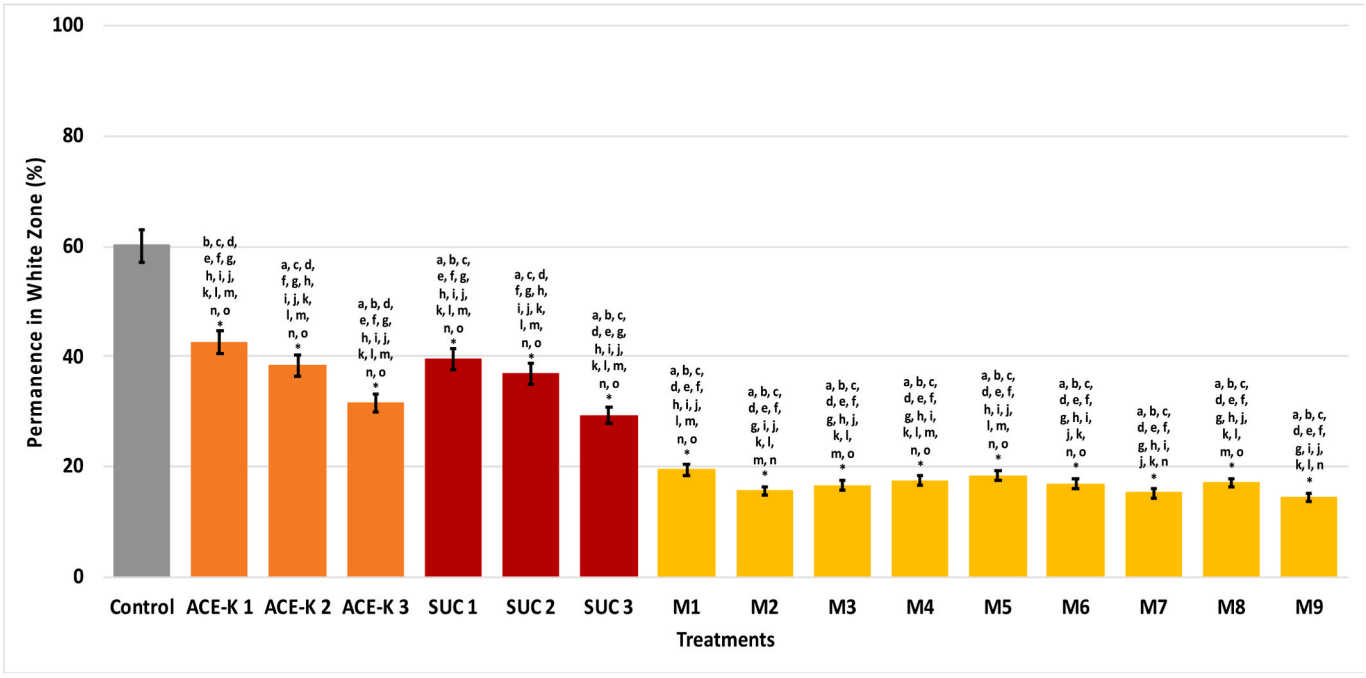


Fig. 1. (*continued*).

noteworthy as fish exposed to SUC, ACE-K, and their mixture spent significantly more time at the top of the aquaria than at the bottom, as compared to the control group. Furthermore, fish exposed to all treatments displayed a higher freezing time at the aquaria's top and bottom than the control group. Concentration-dependent changes were observed in all endpoints, with significant differences between treatments.



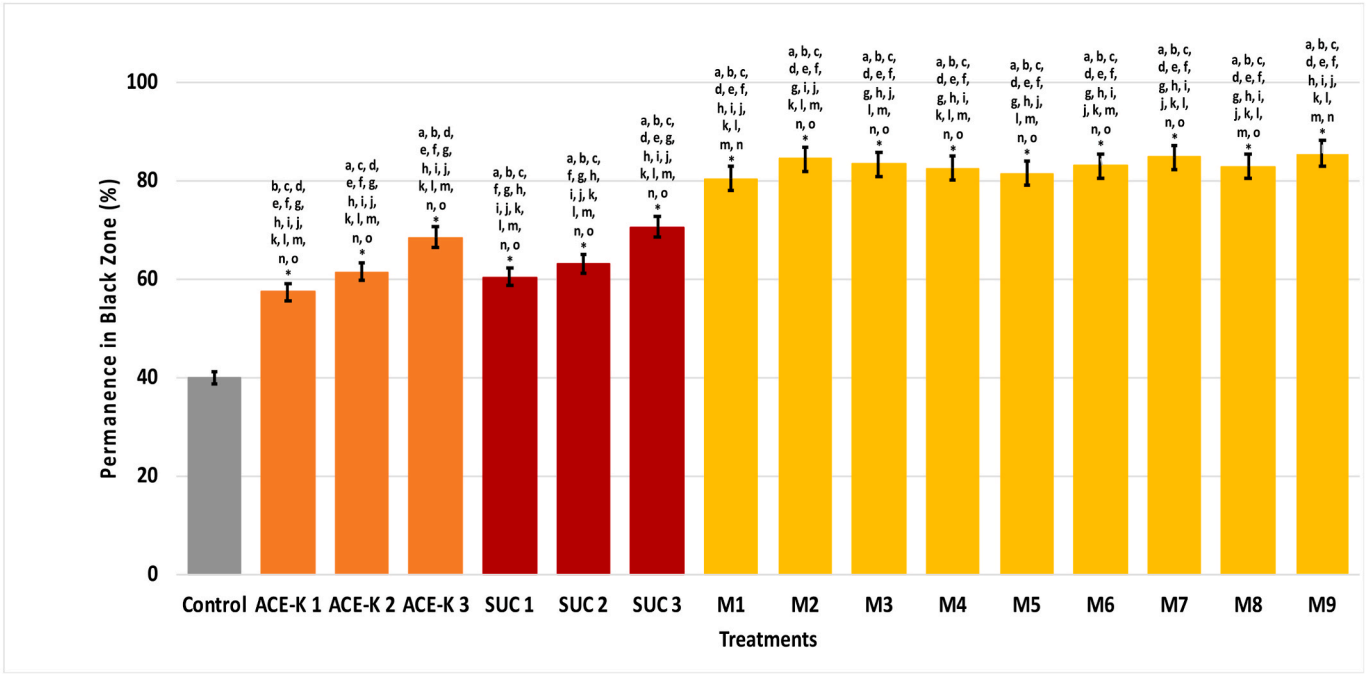


Fig. 2. Dark and light results (A: Permanence in white zone, B: Permanence in Black Zone, C: Freezing Bouts) of fish exposed to SUC, ACE-K, and their mixtures. Data represent the mean ± S.D. of three independent experiments, n = 45. * indicate a significant change compared to the control group. A: indicate a significant change compared ACE-K1. B: indicate a significant change compared ACE-K2. C: indicate a significant change compared ACE-K3. D: indicate a significant change compared SUC1. E: indicate a significant change compared SUC2. F: indicate a significant change compared SUC3. G: indicate a significant change compared M1. H: indicate a significant change compared M2. I: indicate a significant change compared M3. J: indicate a significant change compared M4. K: indicate a significant change compared M5. L: indicate a significant change compared M6. M: indicate a significant change compared M7. N: indicate a significant change compared M8. O: indicate a significant change compared M9.

C

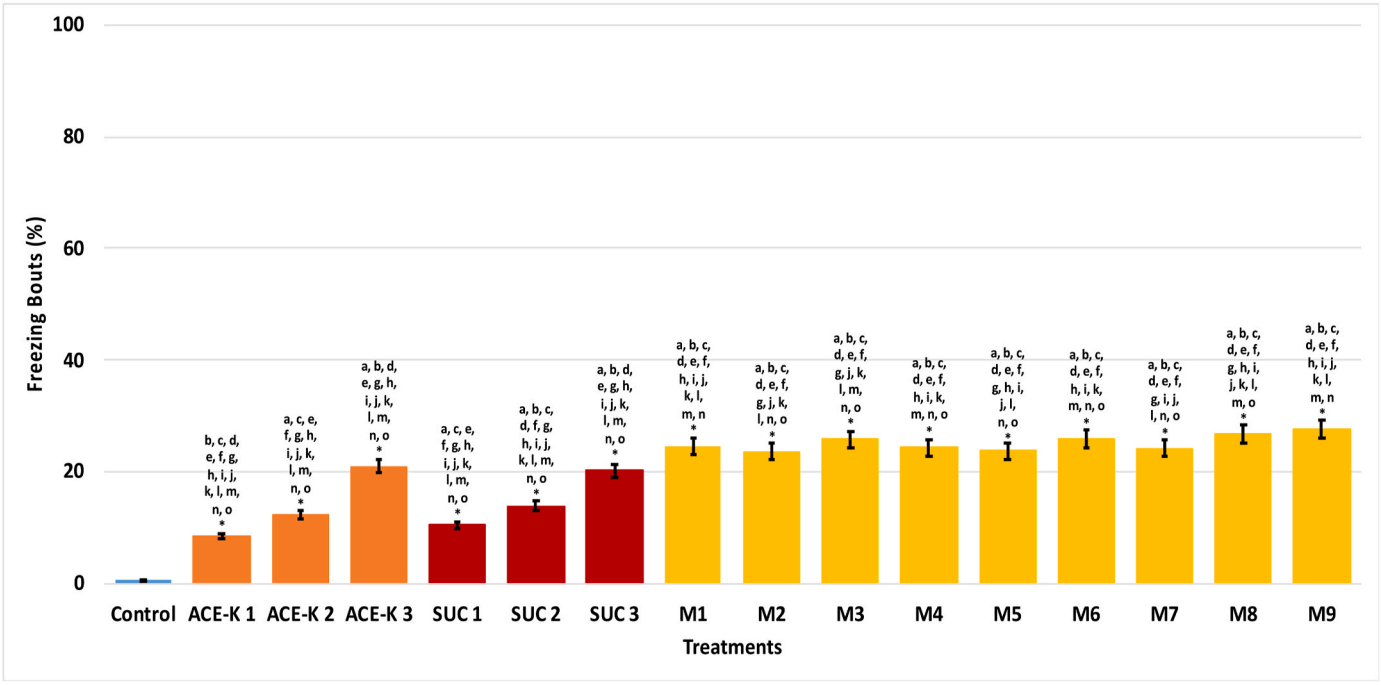


Fig. 2. (*continued*).

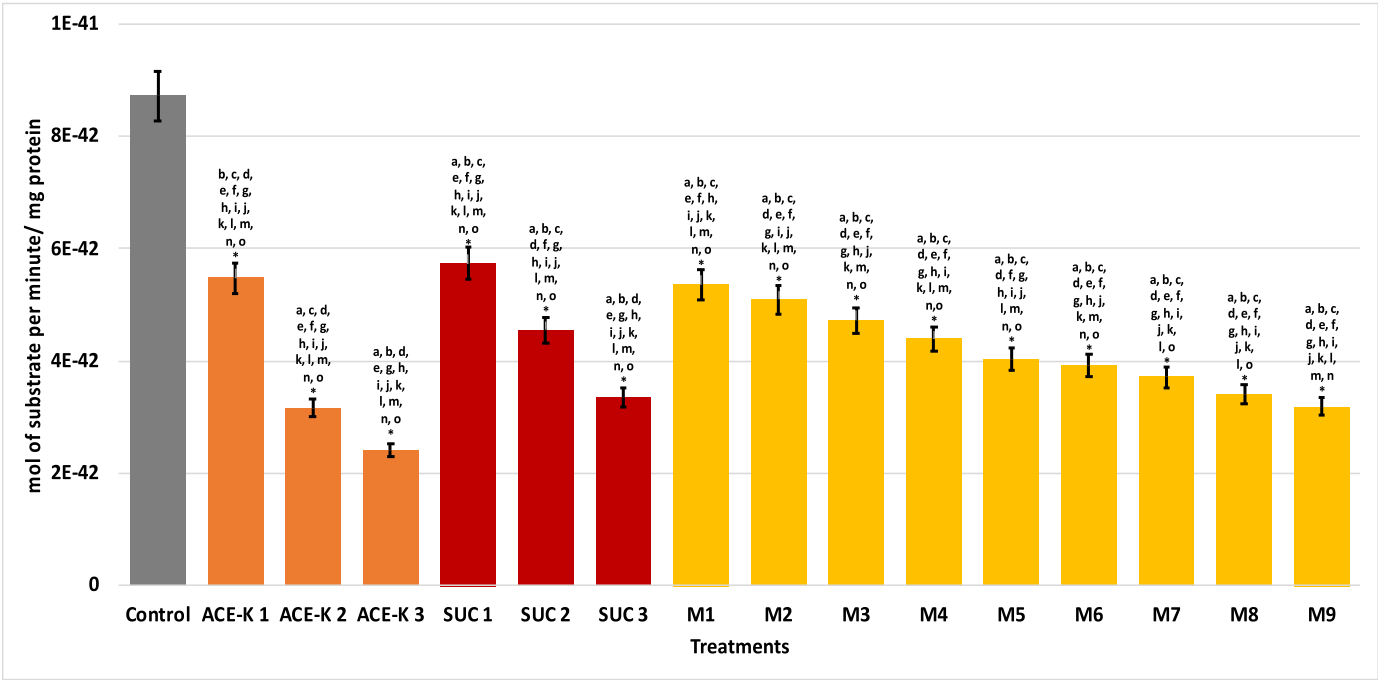


Fig. 3. Inhibition of AChE triggered by SUC, ACE-K, and their mixtures in *Danio rerio* brain. Data represent the mean ± S.D. of three independent experiments, n = 9. * indicate a significant change compared to the control group. A: indicate a significant change compared ACE-K1. B: indicate a significant change compared ACE-K2. C: indicate a significant change compared ACE-K3. D: indicate a significant change compared SUC1. E: indicate a significant change compared SUC2. F: indicate a significant change compared SUC3. G: indicate a significant change compared M1. H: indicate a significant change compared M2. I: indicate a significant change compared M3. J: indicate a significant change compared M4. K: indicate a significant change compared M5. L: indicate a significant change compared M6. M: indicate a significant change compared M7. N: indicate a significant change compared M8. O: indicate a significant change compared M9.

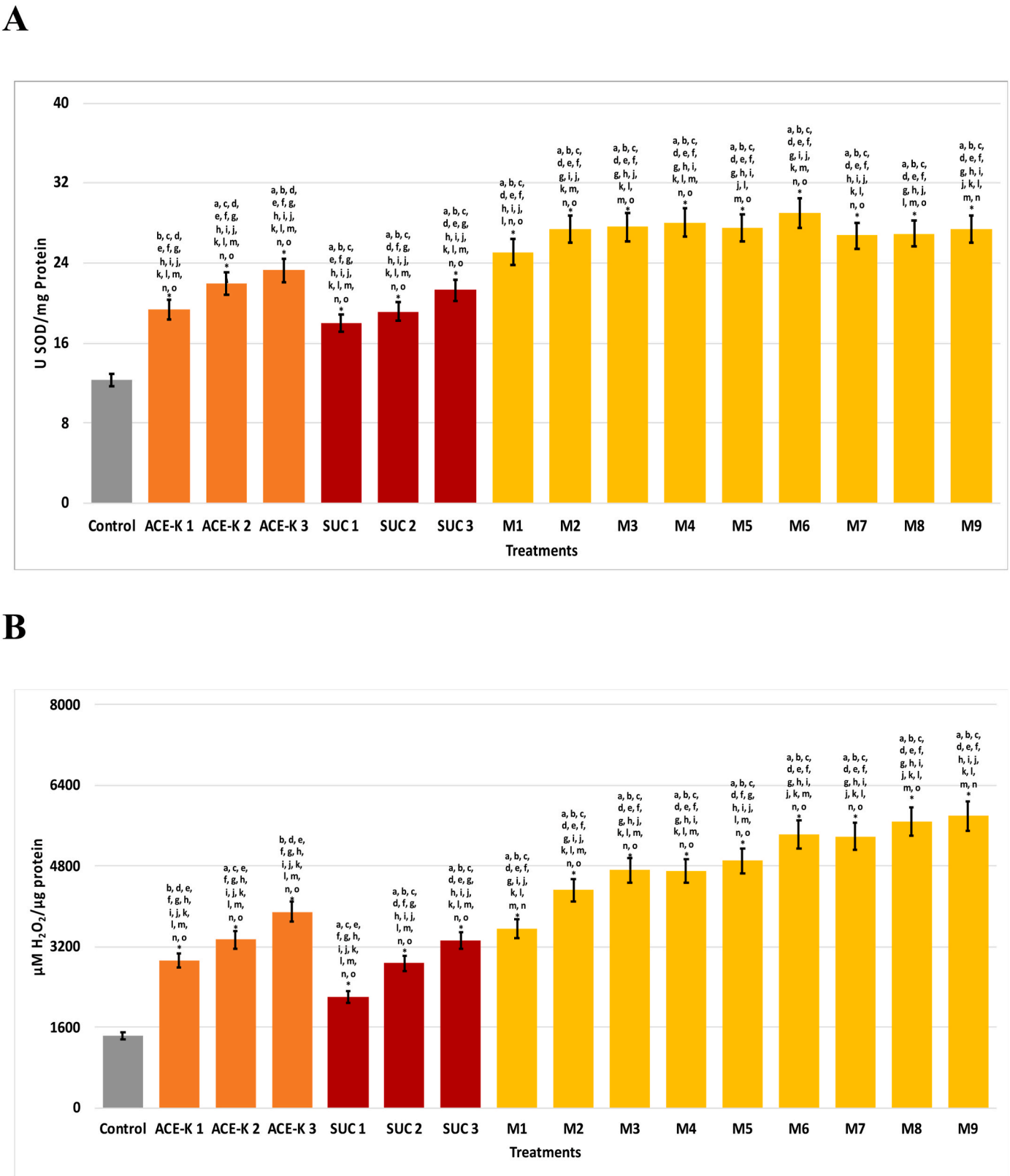
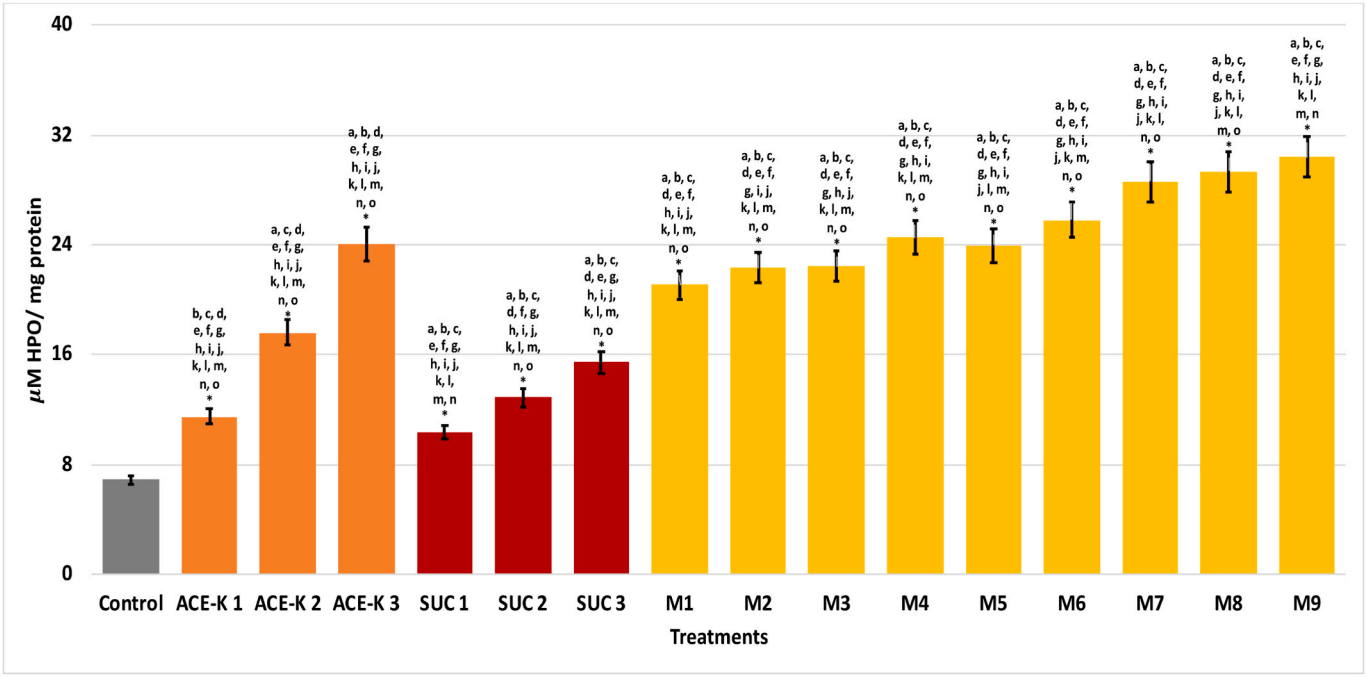


Fig. 4. Oxidative stress (A: SOD, B: CAT, C: HPO, D: PCC, and E: LPO) prompted by SUC, ACE-K, and their mixtures in *Danio rerio* brain. Data represent the mean ± S. D. of three independent experiments, n = 9. * indicate a significant change compared to the control group. A: indicate a significant change compared ACE-K1. B: indicate a significant change compared ACE-K2. C: indicate a significant change compared ACE-K3. D: indicate a significant change compared SUC1. E: indicate a significant change compared SUC2. F: indicate a significant change compared SUC3. G: indicate a significant change compared M1. H: indicate a significant change compared M2. I: indicate a significant change compared M3. J: indicate a significant change compared M4. K: indicate a significant change compared M5. L: indicate a significant change compared M6. M: indicate a significant change compared M7. N: indicate a significant change compared M8. O: indicate a significant change compared M9.

C



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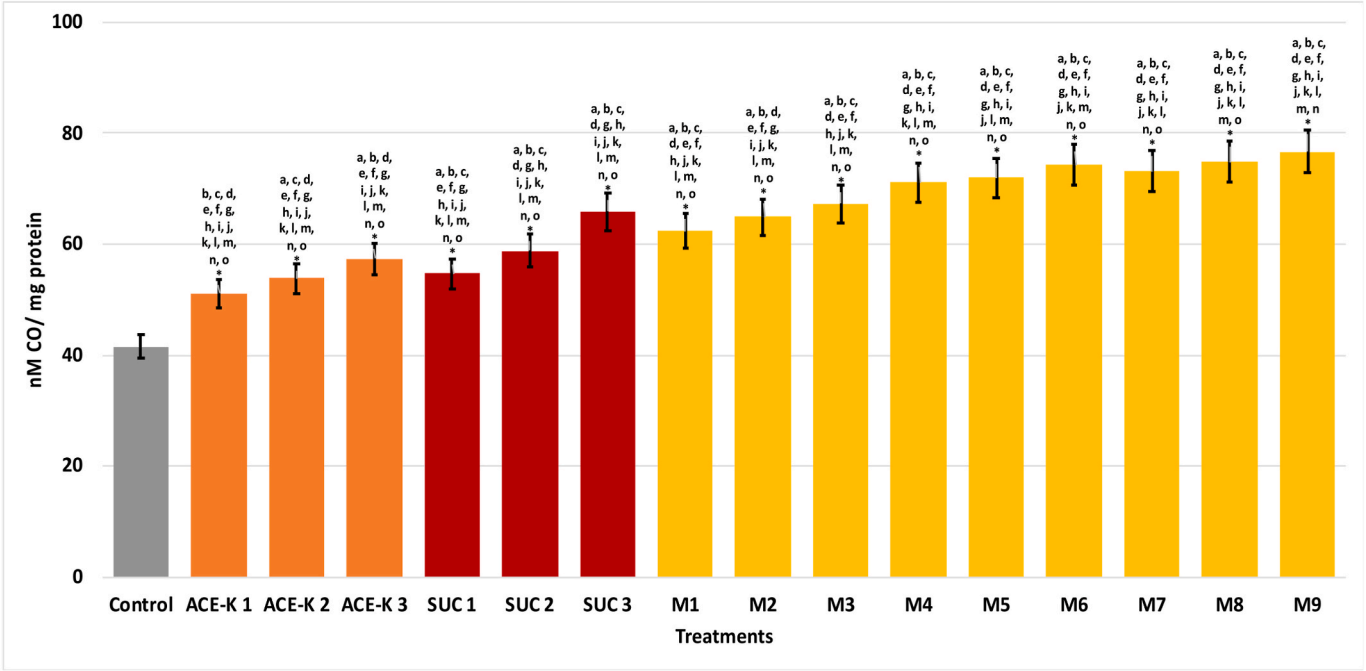

Fig. 4. (*continued*).

3.2. Dark and light test

Following exposure to SUC, ACE-K, and their combinations, the fish exhibited significant alterations in all endpoints evaluated during dark and light tests (Fig. 2 A-C). For instance, fish from all treatment groups spent significantly more time in the dark than in the light area compared to the control group. Additionally, in the Novel Tank test, fish exposed to SUC, ACE-K, and their mixtures displayed an extended period of

E

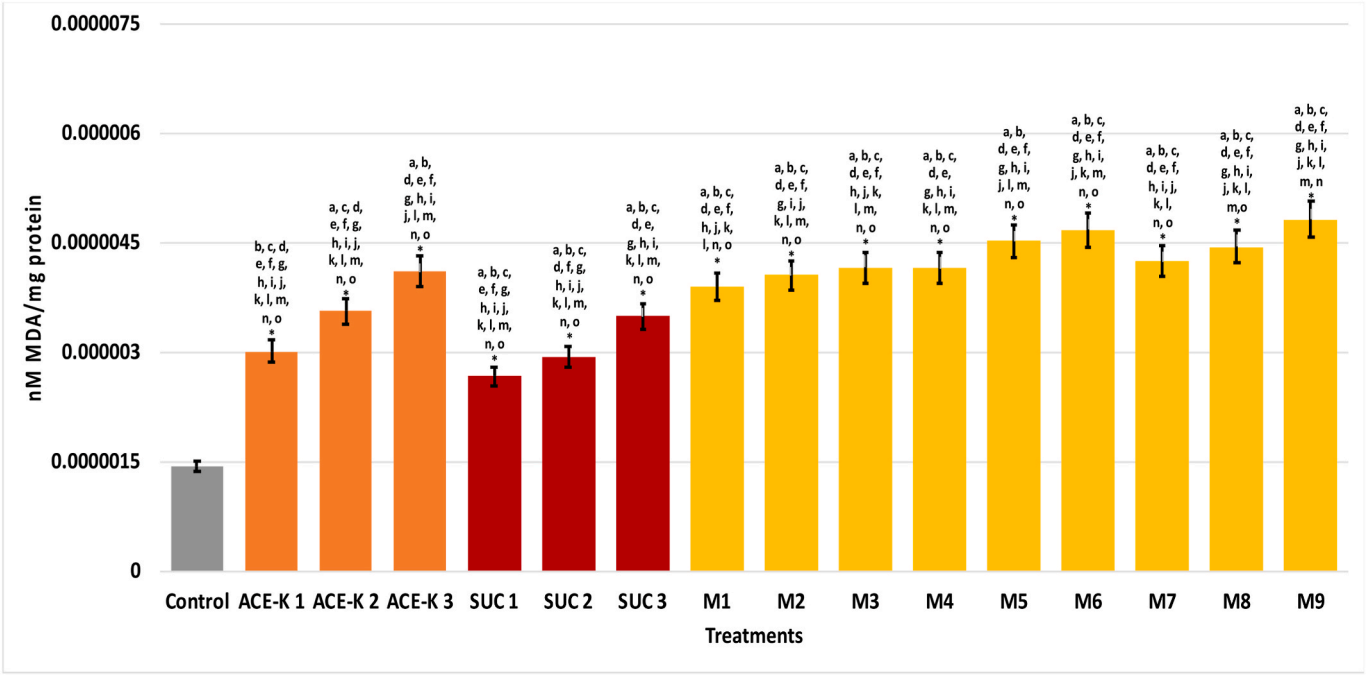

Fig. 4. (*continued*).

immobility compared to the untreated fish. The alterations in individual treatments were contingent on the concentration. However, changes in mixtures were negligible, and no significant differences were observed.

3.3. AChE evaluation

SUC, ACE-K, and their mixtures inhibited the AChE activity of fish in a concentration-dependent manner (Fig. 3). For compounds alone, it is remarkable to mention that the AChE inhibition by ACE-K was more severe than the one produced by SUC. As inhibition of the enzyme was concentration-dependent, we did observe significant differences between treatment groups.

3.4. Oxidative stress

Acute exposure to SUC, ACE-K, and their mixtures induced oxidative stress in the fish brain, as demonstrated in Fig. 4 A-E. The mix of these compounds significantly elevated the levels of all oxidative damage biomarkers in a concentration-dependent manner, akin to the individual compounds. Fish exposed to ACE-K exhibited higher levels of hydroperoxides (HPO) and lipid peroxides (LPO) compared to those exposed to SUC, whereas SUC induced overproduction of protein carbonyls (PCC) in the fish brain. Additionally, exposure to SUC, ACE-K, and their mixture increased the levels of superoxide dismutase (SOD) and catalase (CAT) enzymes in the fish brain. The highest levels of both enzymes were observed in the mixture-exposed fish, followed by those exposed to ACE-K and SUC. Notably, all changes in the oxidative stress biomarkers were concentration-dependent, resulting in significant differences between all treatment groups (see Fig. 5).

3.5. SUC and ACE-K determination

SUC and ACE-K concentrations in the brain in all treatment groups, but the control group, were above the limit of quantification (Table 4). Indeed, concentrations of both compounds increased concentration-dependent in all systems. Thus, SUC and ACE-K crossed the blood-brain barrier and had a detrimental effect on the brain of *Danio rerio*. A noteworthy finding was that we found higher concentrations of ACE-K than SUC in the brain of fish. SUC and ACE-K concentrations in the control group remained below the limit of quantification. On the contrary, the measured concentrations of both compounds in the remaining treatment groups exhibited a decrease; however, the reduction did not surpass 20% when compared to the control group. Table 1S (supplementary material) presents the concentrations of SUC and ACE-K in the water systems (μg/L).

3.6. Response charts and correlogram

From our response charts, it can be appreciated oxidative stress (A-E), freezing (J-L), and permanence in the black zone (O) biomarkers are concentration-dependent and showed a similar response. Moreover, as in most cases, the corners of graphs from the ACE-K response remain in the yellow and red zone, we suggest ACE-K generated a more toxic response than SUC.

Graphs F–I and M, corresponding to AChE activity, distance traveled, and time of permanence in the bottom also showed a similar trend. For all these biomarkers, it can be appreciated that as SUC concentration increases, the response decreases significantly. Meanwhile, for ACE-K, values from all concentrations were held in the blue zone, indicating a more harmful response.

Pheatmap (Fig. 6) depicts the correlation among all variables tested. From it, we can appreciate oxidative stress biomarkers are strongly and poorly correlated to the time fish remained frozen and the period they stayed in the black zone, respectively. Regarding AChE activity, our heatmap denotes that it is positively and strongly correlated to the distance fish swam and the time remaining in the bottom area. Moreover, AChE and oxidative stress biomarkers are negatively correlated, indicating that ROS increase might be related to AChE inhibition.

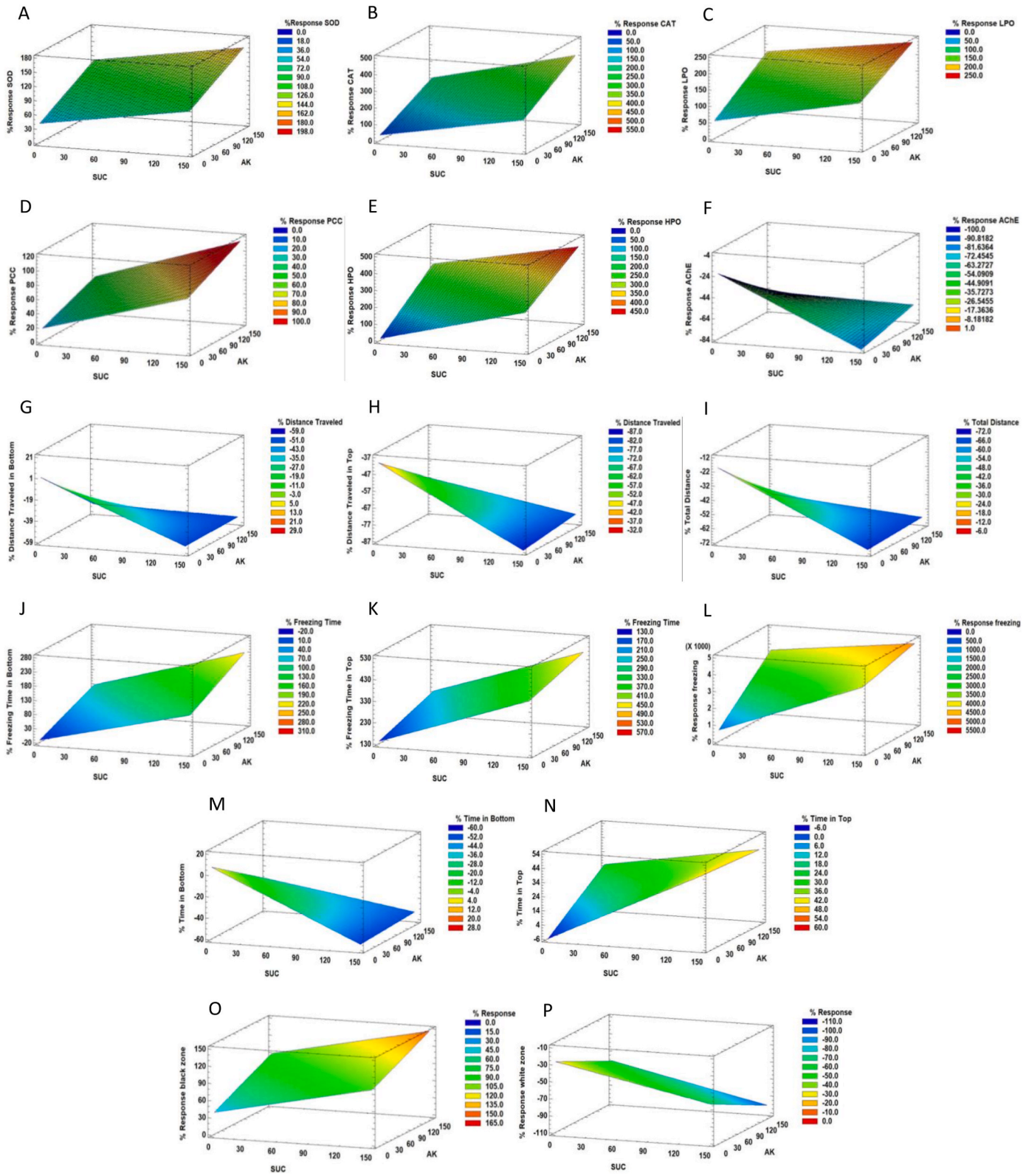

Fig. 5. SUC and ACE-K response charts. Colors denote how intense is the response of both compounds. A to E graphs correspond to oxidative stress biomarkers. G to P are the graphs related to endpoints evaluated during the behavioral test. F reflects the response of both compounds to AChE.

4. Discussion

Previously, only four studies that we are aware of have evaluated the potential oxidative damage SUC may induce in aquatic organisms. Eriksson Wiklund et al. (2014), for instance, demonstrated that 0.0001–5 mg/L of SUC augmented the levels of thiobarbituric acid reactive substances and oxygen radical absorbing capacity in *Daphnia magna* after 24 h. Similarly, Saucedo-Vence et al., 2017; Heredia-García et al. (2019) exposed common carp adults (*Cyprinus carpio*) to 0.05 and 155 μg/L of SUC during four exposures times (12, 24, 48, 72, and 96 h)

Table 4
SUC and ACE-K concentrations in the brain of *Danio rerio*.

Treatments	Nominal Concentrations (μg/L)	Brain Concentrations (μg/g)
Control	Control	<LOQ
SUC 1	50	0.03 ± 0.01
SUC 2	75	0.04 ± 0.01
SUC 3	125	0.07 ± 0.02
ACE-K 1	50	0.21 ± 0.05
ACE-K 2	75	0.34 ± 0.07
ACE-K 3	125	0.63 ± 0.09
M1 (SUC + ACE-K)	50 + 50	0.02 ± 0.01 + 0.24 ± 0.04
M2 (SUC + ACE-K)	50 + 75	0.02 ± 0.01 + 0.33 ± 0.08
M3 (SUC + ACE-K)	50 + 125	0.02 ± 0.01 + 0.62 ± 0.10
M4 (SUC + ACE-K)	75 + 50	0.04 ± 0.01 + 0.22 ± 0.04
M5 (SUC + ACE-K)	75 + 75	0.03 ± 0.01 + 0.31 ± 0.07
M6 (SUC + ACE-K)	75 + 125	0.03 ± 0.01+ 0.66 ± 0.09
M7 (SUC + ACE-K)	125 + 50	0.08 ± 0.02 + 0.21 ± 0.03
M8 (SUC + ACE-K)	125 + 75	0.07 ± 0.02 + 0.30 ± 0.05
M9 (SUC + ACE-K)	125 + 125	0.07 ± 0.02 + 0.65 ± 0.09

Values are the mean of three replicates ± S.D. LOQ-SUC: 13 ng/g LOQ-ACE-K: 17 ng/g.

and observed it increased the levels of lipid peroxidation, hydroperoxides and protein carbonyl content in gills, brain, muscle, and blood of fish. Moreover, in our previous study, results demonstrated that SUC in concentrations ranging from 0.05 to 116 μg/L triggered an enlarged production of reactive oxygen species (ROS) in *Danio rerio* larvae (Colín-García et al., 2022). Concerning ACE-K, information about oxidative damage is even more scarce, as only two studies have reported the effects of this emerging contaminant in aquatic organisms. In the first one, Ren et al. (2016) exposed several *Carassius auratus* fish to 0.1 and 10 mg/L of ACE-K for seven days and saw this sweetener increased malondialdehyde levels and inhibited SOD activity in the liver of fish. Moreover, Cruz-Rojas et al., 2019 indicated that ACE-K at 0.05 and 149 μg/L prompted the production of ROS in the gills, brain, and muscles of *Cyprinus carpio* after 12, 24, 48, 72, and 96 h of exposure. Concordantly with the above information, results from this study indicated that SUC and ACE-K triggered the production of ROS, leading to a remarkable oxidative stress response in the *Danio rerio* brain. Moreover, we also observed that ACE-K oxidative stress response was more intense than the one produced by SUC. In agreement with these results, two previous studies in bacteria and rats indicated that SUC showed comparatively less detrimental effects than ACE-K (Shastry et al., 2012; Shahriar et al., 2020). Thus, at least so far, we can imply that SUC is less toxic than ACE-K; however, more studies in aquatic organisms will help to elucidate this hypothesis. Herein, we did not solely evaluate the oxidative stress of individual compounds but also the disruption to REDOX status triggered by their mixtures, which generated a more severe response than ACE-K and SUC alone. Overall, the mechanism by which these sweeteners prompt oxidative stress in cells remains unknown. However, the scientific community has proposed two mechanisms to fill this knowledge gap. For example, Saucedo-Vence et al. (2017) suggested that the biotransformation process of SUC mediated by the microsomal monooxygenase system and the CYP enzymes could lead to ROS generation. The CYP subfamily CYP3A has specificity over organochlorine drugs; thus, this CYP enzyme could remove the organochlorine sweetener SUC (Schiffman and Rother, 2013). Mora et al., 2021 pointed out that ACE-K can interact with the genetic material of cells. In agreement with this study, Hadidi et al. (2022) used molecular modeling and spectroscopy to demonstrate the formation of the DNA-ACE-K complex, showing ACE-K remained between the guanine 10 and 16 in DNA minor groove. One of the most hazardous DNA lesions is the double-strand break that can lead to apoptosis, inactivation of critical genes, and severe chromosomal aberrations (Barzilai and Yamamoto, 2004). Deactivation of strategic genes can ultimately lead to oxidative stress, as several authors have established (Yang et al., 2005; Semchuk et al., 2009; Seregina et al., 2022).

Oxidative stress is related to cognitive impairment. High levels of polyunsaturated fatty acids, lower brain antioxidant activity, and reduced cellular regeneration capacity make the brain extremely vulnerable to damage by ROS (Chandravanshi et al., 2018). Herein, we demonstrated that SUC, ACE-K, and their mixtures impaired the swimming behavior of *Danio rerio* adults during the dark and light test and the NovelTank test. Fish, from all treatments, denoted an anxiety behavior during both tests, characterized by a significant reduction in the distance

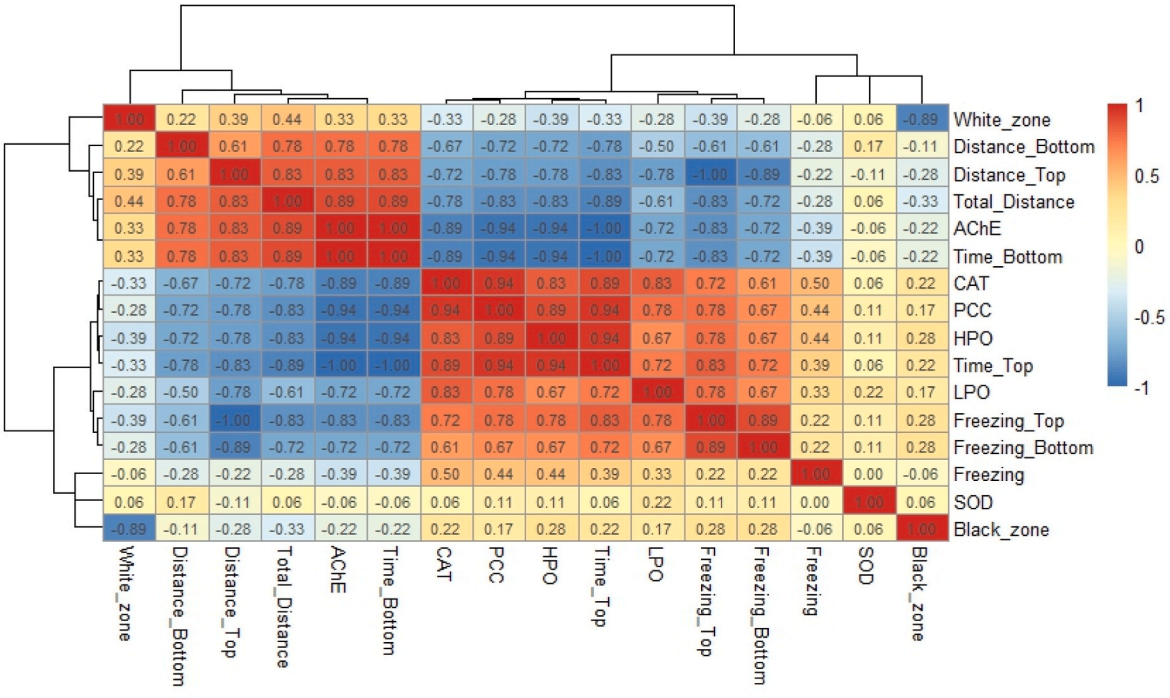

Fig. 6. Pheatmap of biomarkers assessed. Colors denote how intense the relationship of all biomarkers evaluated is.

traveled at the top, a higher freezing time at the top, an increment time of permanence in the black zone, and an increase in the freezing episodes than those exposed to the control solution. As it occurred in oxidative stress, ACE-K altered in a more significant manner the behavior of fish than SUC. Furthermore, fish exposed to mixtures showed more anxiety during swimming than those exposed to compounds alone. Changes in swimming behavior observed in fish during the two trials may also be closely related to acetylcholinesterase (AChE) activity inhibition. AChE is an essential enzyme for organisms as it prevents acetylcholine dispersal and near receptors stimulation for controlling neuronal transmission and signaling between synapses (Trang and Khandhar, 2021). Therefore, inhibition of AChE may lead to paralysis, convulsions, loss of coordination, and other behavioral impairments in organisms (Ren et al., 2015). Moreover, particularly in fish, different studies have associated the inhibition of AChE with the production of anxiety and reduced capacity to escape predation (Sandoval-Herrera et al., 2019; Pullaguri et al., 2020; Elizalde-Velázquez et al., 2022a, 2022b). Even though the AChE-inhibition mechanisms of both compounds have not been studied, we hypothesize it might be the result of the oxidative stress response. For instance, Eriksson Wiklund et al. (2014), observed a positive and negative relationship between AChE and oxidative biomarkers TBARS and ORAC, respectively. Moreover, recent evidence has indicated ROS, H_2O_2 specifically, oxidizes cysteine residues of AChE, affecting the function of this enzyme (Elizalde-Velázquez et al., 2022a, 2022b).

5. Conclusions

Environmentally relevant concentrations of SUC and ACE-K disrupted the REDOX status of the *Danio rerio* brain, triggering a critical oxidative stress response. Furthermore, as oxidative stress in the brain is involved in AChE inhibition, both compounds generated a paramount anxiety behavior in fish. Anxiety behavior, in both cases, was characterized by a significant increase in freezing time and lower swimming activity. Overall, evidence herein suggests ACE-K produced a more intense harmful effect than SUC. However, further studies on aquatic species should elucidate this. Bearing in mind the above results, we suggest EPA should ponder including both compounds on the priority pollutant list.

Credit author statement

Karla Colín-García performed all the exposure experiments. Leobardo Manuel Gómez-Oliván and Gustavo Axel Elizalde-Velázquez were involved in the conception. Leobardo Manuel Gómez-Oliván and Gustavo Axel Elizalde-Velázquez, were involved in the design and interpretation of the data and the writing of the manuscript with input from Sandra García-Medina.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.139928.

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