

## Article

# In vivo Modelling of Toxicity of Eight Commercial Artificial Sweeteners in *Daphnia* Neonates and Zebrafish Embryos through Cardiac Performance Assessments

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**Abstract:** Artificial sweeteners are widely used food ingredients in beverages and drinks to lower calorie intake which in turn prevent lifestyle diseases such as obesity. Epidemiological evidences suggest that an overdose of artificial sweeteners could result to adverse effects after consumption. Thus, our study aims to systematically explore the potential adverse effects of eight commercial artificial sweeteners, including acesulfame-K, alitame, aspartame, sodium cyclamate, dulcin, neotame, saccharin and sucralose on cardiac performances of zebrafish (*Danio rerio*) and *Daphnia* as model animals. Embryonic zebrafish and *Daphnia* were exposed to eight artificial sweeteners at 100 ppb concentrations and their cardiac performance (heart rate, ejection fraction, fractional shortening, stroke volume, cardiac output and heartbeat regularity) were measured and compared. Saccharin significantly increased the heart rate of zebrafish larvae while a significant decrease was observed in *Daphnia*. Significant increase was also noted in zebrafish heart rate variability after incubation in acesulfame K, dulcin, sodium cyclamate, and sucralose. However, a significant increase in *Daphnia* was only observed after incubation in dulcin. Based on Principal Component Analysis (PCA) and hierarchical clustering results, several artificial sweetener samples were species-specific to zebrafish and *Daphnia*. Our study demonstrates the potential adverse physiological effects of artificial sweeteners in cardiovascular systems of zebrafish larvae and *Daphnia*.

**Keywords:** Artificial sweeteners; zebrafish; *Daphnia*; cardiac performance; toxicity

## 1. Introduction

The intake of sugar-sweetened beverages is popularly associated with lifestyle-related diseases such as obesity, type 2 diabetes, and a number of metabolic syndromes [1]. Based on the above disease associations, scientists and healthcare practitioners theorized that non-caloric, high-intensity sweeteners may provide a beneficial alternative in food and beverages. Thus, non-caloric sweeteners replacing natural caloric sweeteners reduces energy density of foods and beverages. However,

whether decreasing energy density in this practice would propel into reduced energy intake, lower body weight, and improved metabolic health is subject to question.

Artificial sweeteners, the non-caloric alternative to sugar, has ironically been linked to consumption of more calories for its ability to increase sugar cravings and dependence as well as impairing caloric compensation leading to appetite stimulation [2]. Independent from this mechanism, artificial sweeteners are associated with impaired glucose tolerance secondary to altered gut microbiota [3,4]. These are some of the proposed mechanisms that despite artificial sweeteners being non-caloric are associated with obesity, type 2 diabetes mellitus and cardiovascular diseases [5]. These associated life-style related diseases are clearly linked to the development of the atherosclerotic plaque which may cause ischemia and acute coronary syndrome [6,7]. However, we theorize that artificial sweeteners may also induce direct cardiac toxicity and damage independent of plaque formation. This is supported by studies done on Wistar albino rats wherein aspartame induces oxidative stress in cardiac muscle and increases heart rate variability [8,9]. Study done in aquatic animal in also showed that aspartame also cause oxidative stress in brain, gills and muscles of common carp [10]. Related diseases and effects of artificial sweetener exposure in human and some animal models are listed below (Table 1)

**Table 1.** Study of related disease and effect on biomarker after artificial sweetener exposure.

	Related disease	Effect on biomarker
<b>Human</b>	Obesity, diabetes, hypertension, cardiovascular event [11], hepatotoxicity [12], nausea, vomiting, thrombocytopenia [13].	Lactate dehydrogenase ↑ [14], Acetylcholinesterase ↓ [15].
<b>Rodent</b>	Obesity, gut biota community shift [4], vascular endothelial dysfunction [16], glucose intolerance [17], thyroid tumor [18] (Rat).	Dopamine ↑, hydroxytryptamine ↑, norepinephrine ↑, epinephrine ↑ [19], Xanthine oxidase ↑, superoxide dismutase ↑, catalase ↑ [8] (Rat).
<b>Fish</b>	Swimming defect, inflammatory in brain and liver [20], and growth malformation [21] (Zebrafish).	Reactive oxygen species ↑ [20] (Zebrafish), superoxide dismutase ↑, catalase ↑, lipid peroxidase ↑ [10] (Common Carp).
<b>Daphnia</b>	No (directly linked to mortality).	Lipid peroxidation ↑, AChE ↑, antioxidant capacity ↑ [22].

\* ↑ mean increase and ↓ mean decrease.

Understanding the pathophysiologic mechanism of artificial sweeteners on cardiac toxicity requires models wherein the parameters of interest are easily measured but at the same time represent the complexity of the human heart. *Danio rerio*, also known as the zebrafish, is a commonly used vertebrate model in physiologic, genetic and regenerative experiments on cardiac diseases [23-26]. Among the vertebrate species, it is particularly favored because of its relatively short life span which allows investigators to monitor the disease in an accelerated phase [27]. Another organism, *Daphnia magna*, is an established invertebrate model for the investigation of toxins in water in ecologic systems [28,29]. This invertebrate can also be used as a model for cardiovascular diseases for it has a myogenic heart which exhibits responses comparable to the human heart [30].

As part of our interests to provide *in vivo* models to study the effects of substances in a variety of disease model systems [31-33], zebrafish and daphnia were explored as animal host models to investigate cardiac performance after exposure to eight different artificial sweeteners by measuring different cardiac parameters such as the stroke volume, cardiac output, ejection fraction, and shortening fraction, along with heart rate, heartbeat interval, and heart rate variability.

## 2. Materials and Methods

2.1. Animal ethics and artificial sweetener exposure

All protocols and procedures involving zebrafish were approved by the Committee for Animal Experimentation of the Chung Yuan Christian University (Approval No. 109001, issue date 15 January 2020). In this study, wild type AB strain zebrafish was used as a vertebrate model and maintained in a continuously filtered and aerated water system. Temperature was maintained at 26 °C with a 10/14 hours of dark/light cycle according to previously reported protocols [34]. The eggs were collected and kept in an incubator at 28°C until the time of treatment. At 60 hour-post-fertilization (hpf), approximately fifteen zebrafish larvae were exposed to 20 mL volume of artificial sweeteners at 100 ppb doses in a 9 cm Petri dish. At 72 hpf, zebrafish heartbeat at the ventricle chamber was recorded (Fig. 1A). *D. magna* was used as an invertebrate model. Female *Daphnia* neonates were collected when body size grew around 0.7-2 mm<sup>2</sup> and exposed to artificial sweeteners at 100 ppb concentration at 26 °C for 24 hours. After incubation, the heartbeat at the heart chamber of *Daphnia* was also recorded (Fig. 1B).

In this study, eight artificial sweeteners acesulfame K, alitame, aspartame, dulcin, neotame, saccharin, sodium cyclamate, and sucralose were tested for their potential cardiotoxicity in zebrafish. All of the artificial sweeteners were purchased from Aladdin chemicals (Aladdin, Shanghai, China). Stock solution of artificial sweeteners was prepared with distilled water and diluted to a stock concentration of 1000 ppm. During the assay, stock solutions were diluted to a testing concentration of 100 ppb and applied to embryonic zebrafish and *Daphnia* (Table 2). The assay concentration 100 ppb was shown to be the lowest concentration that can alter cardiac parameters based on our pre-testing protocols. MS222 (Tricaine methanesulfonate) and glucose were used as negative and positive control, respectively. MS222 has been used as anesthetic for zebrafish and *Daphnia*, and is known to decrease cardiac performance [35,36] while glucose known to increase cardiac parameter [37,38]. The experiment was done in triplication with average of five individuals per compound.

Table 2. Molecular formula and acute toxicity information of the eight artificial sweeteners.

Number	Artificial sweetener	Molecular formula	Aquatic acute toxicity
1	acesulfame K	C <sub>4</sub> H <sub>4</sub> KNO <sub>4</sub> S	LC50: 96 Hr for Fish: (mg/L): > 1000
2	alitame	C <sub>14</sub> H <sub>25</sub> N <sub>3</sub> O <sub>4</sub> S	N.A.
3	aspartame	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O <sub>5</sub>	N.A.
4	dulcin	C <sub>9</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>	N.A.
5	neotame	C <sub>20</sub> H <sub>30</sub> N <sub>2</sub> O <sub>5</sub>	N.A.
6	saccharin	C <sub>7</sub> H <sub>5</sub> NO <sub>3</sub> S	N.A.
7	sodium cyclamate	C <sub>6</sub> H <sub>12</sub> NO <sub>3</sub> SNa	N.A.
8	sucralose	C <sub>12</sub> H <sub>19</sub> Cl <sub>3</sub> O <sub>8</sub>	N.A.

WHO GHS acute aquatic toxicity definition is 96hr LC50 less than 1 ppm for fish or 48 hr EC50 less than 1ppm for Crustaceans. N.A. not available.

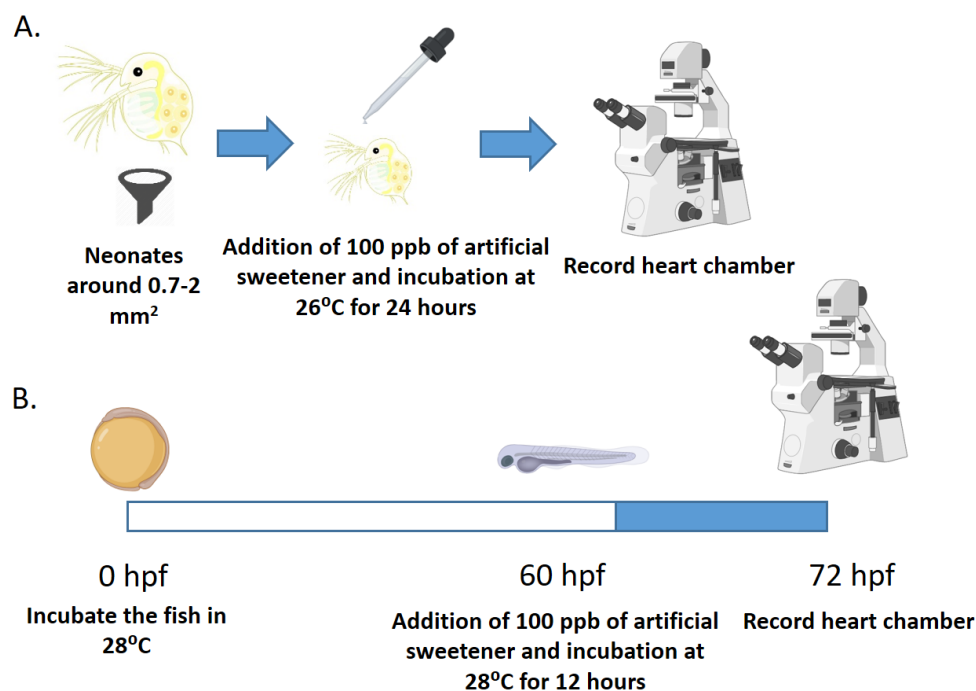
2.2. Cardiac Performance measurement

To record zebrafish heart beats and cardiac physiology parameters, 3% methyl cellulose was used as a mounting agent to minimize zebrafish movement during video recording. High-speed digital charged coupling device (CCD) (AZ Instrument, Taichung, Taiwan) was mounted on inverted microscope (Sunny Optical Technology, Zhejiang, China) to record zebrafish heartbeat. To acquire better image contrasts and resolutions, Hoffmann objective lens with 40x magnification were used, and video was recorded at 200 frames per second (fps) for 10 second [39]. Similar settings were also used to record *Daphnia* heart with LPlan Lens with 20x magnification to record heart rate. To calculate cardiac parameters, Time Series Analyzer V3 plug-in (<https://imagej.nih.gov/ij/plugins/time-series.html>) on ImageJ software was used to analyze the pattern of changes in dynamic pixel intensity [40,41]. Heart rate, expressed as beats per minute (bpm), was measured using the Peak analyzer function in OriginPro 2019 software (Originlab Corporation, Northampton, MA, USA) by

determining the time interval of each peak. The Poincare Plot was generated using a Poincare plug-in from the OriginPro 2019 software. Sd1 and sd2 extracted from the plots were recorded and statistically analyzed to calculate heart rate. Stroke volume is determined by the assumption that heart chamber have ellipsoid shape and calculated by subtracting end-systolic volume (ESV) from the end-diastolic volume (EDV). The volume of the heart chamber was calculated using the heart chamber long ( $D_L$ ) and short axis ( $D_S$ ) to compare the volume difference between EDV and ESV [42]. Cardiac output was calculated by multiplying heart rate observed in the ventricle with stroke volume. Ejection fraction and shortening fraction was calculated using the formula:

$$EF(\%) = \frac{SV}{EDV} \times 100\%$$

$$SF(\%) = \frac{D_s(EDV) - D_s(ESV)}{D_s(EDV)} \times 100\%$$



**Figure 1.** Schematic diagram showing experimental design on testing cardiotoxicity of eight artificial sweeteners in *Daphnia* neonates (A) and zebrafish embryos (B).

### 2.3. Principal component analysis (PCA) and hierarchy clustering

Principal component analysis (PCA) and the heat map are two methods that able to reduce data complexity and perform data grouping. PCA and heat map were constructed using a ClustVis online tool (a web tool for visualizing clustering of multivariate data, <https://biit.cs.ut.ee/clustvis/>) with default setting [43]. To obtain accurate clustering, data were normalized with the control and mean percentage was calculated.

### 2.4. Biostatistics

Statistical analysis was done by latest GraphPad Prism (GraphPad Inc., La Jolla, CA, USA). To determine the significance, non-parametric One-way ANOVA (Kruskal–Wallis test) was performed and the level of significance was set at  $p$  value < 0.05.

## 3. Results

### 3.1. Cardiac performance in zebrafish and *Daphnia* after exposed to artificial sweeteners

To assess cardiac performance, heart rate, cardiac interval, stroke volume, cardiac output, ejection fraction, and shortening fraction were analyzed using ImageJ software. Heart rate variability was calculated using a Poincare Plot to check regularities of each beat after exposure to different artificial sweeteners [44].

The cardiac cycle is divided into two phases: systole and diastole [45]. Increases in heart rate affect diastole by decreasing the time that it takes for blood to fill the ventricles. Conversely, at slower heart rates, left ventricular end diastolic volume is larger. It is also during diastole that blood flows to the coronary arteries to supply the heart [46]. Increases in heart rate will decrease coronary perfusion time and this is problematic in patients with coronary stenosis for it can lead to tissue ischemia. Furthermore, elevation of heart rate adversely affects myocardial oxygen balance by increasing the demand for oxygen [47]. Increases in heart rate also adversely affects patients with pulmonary hypertension since it worsens biventricular function [48]. Hence, artificial sweeteners that cause increase in heart rate should not be given to patients with coronary artery stenosis for it will further lead to tissue ischemia.

Heart rate can be affected by hormones, temperature, or by exogenous compounds [49]. At 100 ppb test concentration, acesulfame K, neotame, saccharin, and sucralose significantly increased zebrafish heart rate compared to the control group (Fig. 2A). On the other hand, saccharin significantly decreased heart rate in *Daphnia* (Fig. 2B).

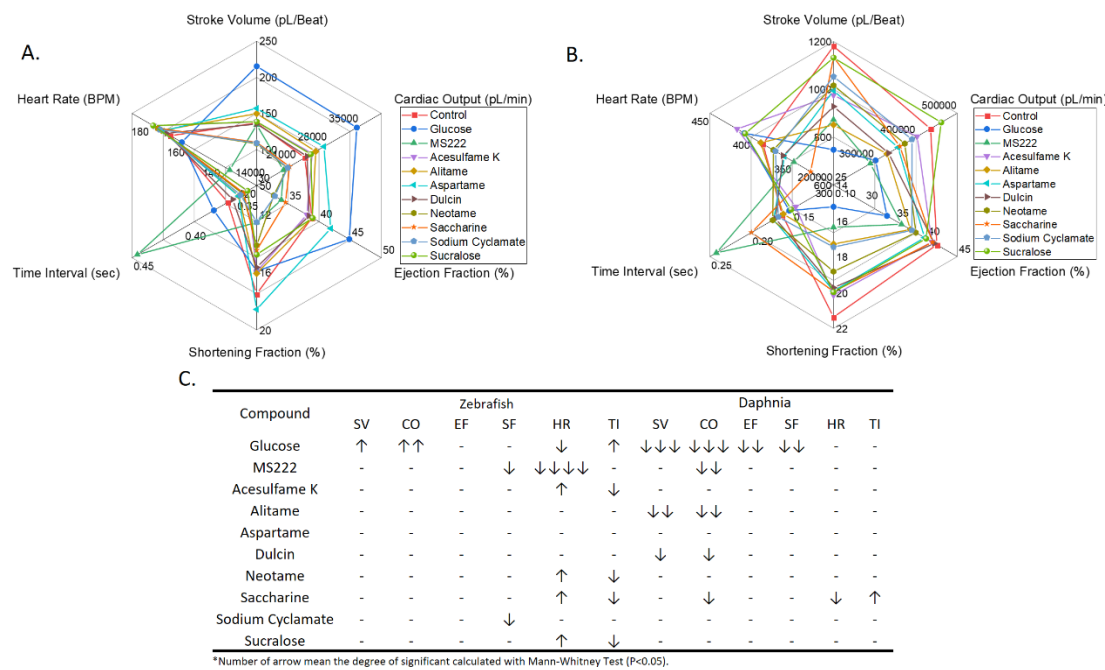
Low time intervals, as noted in increased heart rate, result in a reduced stroke volume secondary to a decrease in ventricular filling. Humans, particularly athletes used to high intensity exercise, will manifest a longer time interval between each beat allowing ventricles to fill with blood efficiently [50]. After incubation with 100 ppb test concentration of acesulfame K, neotame, saccharin, and sucralose in zebrafish larvae, a significant decrease in each beat was observed (Fig. 2A). In *Daphnia*, significant increase in time interval was noted after incubation in 100 ppb of sucralose (Fig. 2B).

To measure the amount of blood pumped during systole, stroke volume was also determined [39]. Stroke volume was calculated by subtracting the volume of the heart chamber at end diastolic phase (EDV) with the heart chamber volume at end systolic phase (ESV). No significant change in zebrafish stroke volume after incubation in all compounds was observed (Fig. 2A). However, a significant decrease was observed after incubation in alitame and dulcin in *Daphnia* (Fig. 2B).

Cardiac output, an important end point in cardiovascular system assessment, was determined by multiplying heart rate and stroke volume [51]. Thus, incubation in acesulfame K, alitame, aspartame, dulcin, and sucralose slightly elevated cardiac output in zebrafish compared to the control group (Fig 2A). The opposite was true in *Daphnia*, where significant decrease in cardiac output compared to the control group after incubation in alitame, dulcin, and saccharine was observed (Fig. 2B).

Ejection fraction was likewise calculated by dividing stroke volume from EDV [52]. No significant change in the ejection fraction among the different artificial sweeteners used on either zebrafish or *Daphnia* compared to the control were observed (Fig. 2A and 2B).

Finally, the index of shortening fraction was determined by obtaining the ratio of the length of heart chamber at the end of systolic phase to end of diastolic phase, which represents muscular contractility of the heart [52]. The artificial sweetener sodium cyclamate significantly decreased the shortening fraction in zebrafish (Fig. 2A). In contrast, all other sweeteners decreased the shortening fraction in *Daphnia* (Fig. 2B).

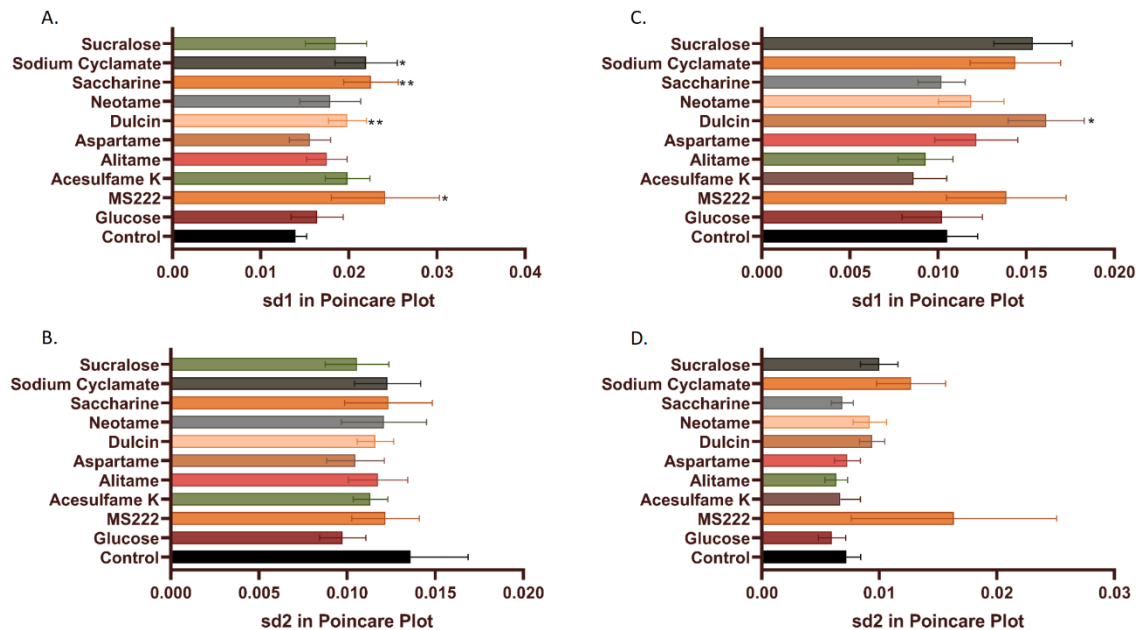


**Figure 2.** Radiac map comparing the mean value of cardiac performance endpoints in either zebrafish (A) or *Daphnia* (B) after exposure to eight different artificial sweeteners. (C) The degree of significance of each cardiac performance endpoints in either zebrafish or *Daphnia* after exposure to eight artificial sweeteners. SV, stroke volume; CO, cardiac output; EF, ejection fractioning; SF, shortening fraction; HR, heart rate and TI, time interval.

3.2. Heart rate regularity in zebrafish and Daphnia after exposure to artificial sweeteners

Using Poincare plot, the artificial sweeteners were checked whether they confer alteration in the variability of heart rate. Poincare plot is a method that plots the heartbeat interval between two successive heart beats and has been used to study heart rate regularity in humans, rodents and zebrafishes [53-56]. A higher standard deviation means that the heartbeat is more irregular. Thus, incubation of zebrafish larvae in acesulfame K, dulcin, saccharin, and sodium cyclamate significantly increased sd1 but showed no significant difference with sd2 (Fig. 3A&B). While slightly elevation of heart rate variability after incubation in neotame, sodium cyclamate, and sucralose in *Daphnia* was observed, however not reach statistical significance. Among eight artificial sweeteners tested, only dulcin significantly increased the heart rate variability with significant higher sd1 value in *Daphnia* (Fig. 3C & D).

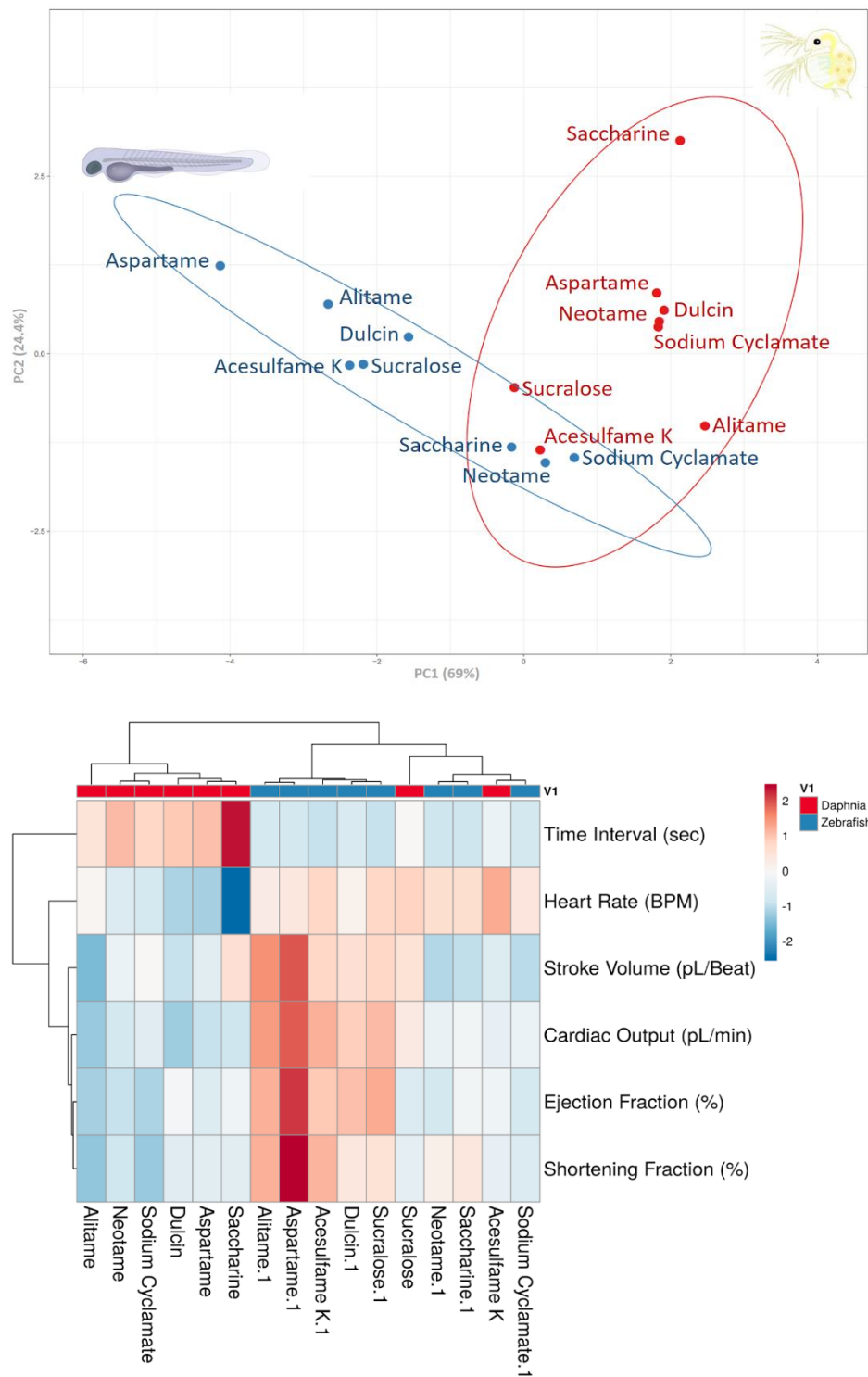




**Figure 3.** Comparison of heart rate regularity in zebrafish embryos (A&B) and *Daphnia* (C&D) after incubation with eight different artificial sweeteners. Data analysis was based on the standard deviation generated by Poincare Plot, and presented as mean  $\pm$  SEM and statistical significances were tested by non-parametric One-Way ANOVA (Kruskal–Wallis test) with post-hoc analyzed. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

### 3.3. Comparison of cardiac performance between zebrafish and *Daphnia* after exposure to artificial sweeteners

In the PCA (principal component analysis) and heat map analysis, sucralose and acesulfame K in *Daphnia* showed closer clustering with sodium cyclamate, saccharine, and neotame in zebrafish (Fig. 4). Unlike the other compounds, acesulfame K and sucralose in *Daphnia* increase the heart rate while the other compound decreased the heart rate of *daphnia* and these effects also observed in zebrafish. Clustering analysis resulted into three groups. The first group comprised of aspartame, alitame, dulcin, neotame, sodium cyclamate, and saccharin showed specific effects on *Daphnia*. The second group consisting of acesulfame K, alitame, aspartame, dulcin, and sucralose showed similar effects in zebrafish. Finally, the third group which includes sucralose and acesulfame K in *Daphnia* and saccharine, neotame, and sodium cyclamate in zebrafish as they have similar effect in altering cardiac parameter endpoint we tested.



**Figure 4.** Principal component analysis (Top panel) and Heat map (Bottom panel) for cardiac physiological endpoint alterations in either *Daphnia* (red color) or zebrafish (blue color) after exposure to eight different artificial sweeteners. Results show the correlation between every compound tested in every endpoint. The cardiac physiology endpoints including SV, stroke volume; CO, cardiac output; EF, ejection fractioning; SF, shortening fraction; HR, heart rate and TI, time interval.

4. Discussion

4.1. The importance of using zebrafish and *Daphnia* to study artificial sweetener adverse effects on cardiac performance



The effects of a number of substances in aquatic animals such as zebrafish, medaka, *Daphnia* and *Xenopus* have long been investigated [57-61]. In order to address the full spectrum of toxicity of different substances, studies often utilize multiple aquatic models [62]. Invertebrates such as *Daphnia* are used for initial toxicity screening followed by more complex organisms such as zebrafish for organ-specific toxicity assessment [62]. Inspired by this idea, we used both *Daphnia* and zebrafish to test the effects of artificial sweeteners on cardiac performance parameters.

Based on the PCA and heat map plots, we observed that alitame, aspartame, and dulcin showed species-specific effects. While receptor-specific binding tests were not performed, we proposed that *Daphnia* and zebrafish might have different receptors for artificial sweetener ligands. Moreover, in cases where different species have similar receptors for the same ligand, receptors may have different conformations resulting in different responses. This can happen even among species within the similar phylogenetic tree [63-65]. For example, a previous study focused on taste receptors in primates demonstrated that glycine, D-phenylalanine, D-tryptophan, cyanosouan, magapame, and sucronate can induce sweet taste buds to all primates while in contrast, campame, cyclamate, and superaspartame induced sweet taste buds only in a few primates [66]. In another study testing pain sensitivity of human show that humans have difference sensitivity to pain because of the variance of pain receptor [67]. These studies suggest that some stimuli have different effects on different species. Therefore, our findings clearly demonstrate zebrafish and *Daphnia* with distinct response toward artificial sweeteners on cardiac performance, also support that multiple animal model is required to fully recapitulate the potential adverse effect for artificial sweeteners in vivo.

#### 4.2. Saccharin induces distinct cardiac performance in zebrafish and *Daphnia*

The most important finding in this study is significant alteration of heart rate and heartbeat interval in zebrafish and *Daphnia* in an opposite manner by saccharin. A previous study showed that saccharin treatment to rat caused oxidative stress in brain by increasing MDA (Malondialdehyde) level [68]. In previous study, zebrafish treated with saccharin also exhibited infiltration of inflammatory cells into zebrafish liver and brain by increasing cholesteryl ester transfer protein, which play an important role in transporting and exchange of cholesteryl ester to triglyceride and *vice versa* [20]. Heart rate is controlled by signals from the brain, therefore, damage in the brain may cause alterations in heart rate regulation [69-71].

It was also reported that saccharin reduced total antioxidant capacity (TAC) and catalase activity and caused liver damage in rats [72]. Furthermore, a separate study also described that saccharin can damage hepatic and renal tissue and significantly decrease catalase, glutathione, and superoxide dismutase levels in rat liver [73]. Decrease in antioxidant and catalase activity led to the increase of reactive oxygen species levels, which caused tissue damage, especially in sensitive organs such as the heart. In this study, saccharin altered heart rate in both *Daphnia* and zebrafish. While no evidence of physical damage in the heart was observed in both species, we hypothesize that oxidative stress may lead to organ damage in longer exposure times. Evidence also showed that incubation in saccharin also caused increase in heart rate variability. While not significant in *Daphnia*, there was a significant increase in zebrafish heart rate variability which strengthened the possibility of heart damage (Fig. 3).

#### 4.3. Possible mechanism of artificial sweetener on modulating cardiac performance in zebrafish and *Daphnia*

Cardiac function is mediated by the sympathetic and parasympathetic nervous systems [74]. The sympathetic nervous system acts on adrenergic receptors (AR) which are G-Protein coupled receptors (GPCR) [75]. Stimulation of  $\beta_1$ ARs and  $\beta_2$ ARs in the heart-increases cardiac contractility, frequency, rate of relaxation, acceleration of impulse conduction through the atrioventricular node as well as increased pacemaker activity from the sinoatrial node by increasing intracellular  $\text{Ca}^{2+}$  concentration [76]. Previous studies reported that aspartame can increase brain adrenergic neurotransmitters in various parts of the mouse brain [77]. Furthermore, aspartame increases sympathetic activity within half an hour after consumption either in the form of diluted water or in aspartame-sweetened diet drink in humans [78]. These findings suggest the possibility of artificial sweeteners in altering the

sympathetic nervous system, and finally induce cardiac performance alteration in zebrafish and *Daphnia*.

After incubation in acesulfame K, neotame, saccharin, and sucralose, heart rate was significantly elevated in zebrafish. Dopamine is one of hormones that also acts as a neurotransmitter which functions primarily in the central nervous system and usually related to happiness. The role of dopamine on mediating food reward and stimulating palatability is well-established. Sucrose induces dopamine release in rats [79]. Also, the uptake of saccharin increases dopamine levels in rats [80]. Among the reasons for the increase of dopamine release is related with the food reward system that is induced by sweet taste [81]. The positive effect of dopamine on heart rate and muscle contractility has been observed in animal models, such as dogs [82] and rats [83], thus corroborating to our results that some artificial sweeteners can increase heart rate.

T1R and T2R are receptors expressing on taste buds that belong to a superfamily of GPCRs mediating sweet stimuli in humans and are highly expressed in the olfactory system especially in the tongue [84,85]. Those receptors have also been isolated and characterized in fish as well, which share high conservativity to human T1R and T2R counterparts [86]. T1R homolog in zebrafish responds to artificial sweeteners by increasing dopamine concentration [87]. However, although the whole genome of *Daphnia magna* has been decoded, no report of T1R/T2R taste receptor homologs are found in *Daphnia* and study by Penalva-Arana et al. suggest that taste response in daphnia mediated by gustatory receptor superfamily (Grs) [88]. Therefore, we proposed this might be one of the reasons to explain why some artificial sweeteners increase heart rate variability in zebrafish larvae but not in *Daphnia*.

$\gamma$ -aminobutyric acid (GABA) is one of the neurotransmitters that plays an important role in inhibiting neuronal activity. In vertebrates, the heart rate is controlled by GABA signaling, including in *Daphnia* and zebrafish [89,90]. For example, the activity of dopamine D<sub>2</sub> receptor modulated AKT signaling and altered GABAergic neuron development and motor behavior in zebrafish larvae [91]. Addition of dopamine significantly increased the variability of sd1 in zebrafish larvae [92]. Moreover, GABA receptor homolog in *Daphnia pulex* showed similar function [93]. Therefore, we hypothesized the heart rate in both zebrafish and *Daphnia* is mediated by GABA system, and the administration of artificial sweeteners can trigger similar heart rate variability in zebrafish larvae, yet the lack of sweet receptors diminished the phenomena in *Daphnia*.

At the preliminary testing, we found that *Daphnia* stroke volume and cardiac output have unusually high variation rate. We hypothesize that body size has a positive correlation with heart size and this variation might become a potential bias in accurately assessing 2D video imaging based methods [94]. This problem is not only seen in the assessment of cardiac performance but also affects total protein and other biomarkers in *Daphnia* [95]. To solve this problem, we normalized the stroke volume and cardiac output by measuring *Daphnia* body area. Our study also suggests that normalizing data by body size provides a more reliable index especially in cardiac performance analysis [96]. In the future, normalization of stroke volume and cardiac output with the body size should become a good method on accurate assessing cardiac parameters in *Daphnia*.

## 5. Conclusions

Our study showed the potential adverse effects on cardiac physiology in zebrafish larvae and *Daphnia* neonates after exposure to commercial artificial sweeteners. Although no significant phenotypic change (like edema) was observed during the experiment, our study demonstrated that acute exposures to artificial sweeteners at low concentration (100 ppb) indeed can alter cardiac performance in two animal models. Our PCA analysis also demonstrated species-specific effects between zebrafish and *Daphnia*, highlighting the importance of using a variety of animal models in assessing toxicities of chemical compounds.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

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