

Effects of 432 Hz and 440 Hz Sound Frequencies on the Heart Rate, Egg Number and Survival Parameters in Water Flea (*Daphnia magna*)

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ABSTRACT

The noise pollution caused by anthropogenic activities in the aquatic environment negatively affects aquatic organisms like mammals, fish, crustaceans, and even plankton, which are important for the aquatic ecosystem. This study was conducted to examine the effect of lethal and sub-lethal effects of high-frequency sound exposure on *Daphnia magna* aiming to obtain more detailed scientific information. For this purpose, a total of 150 gynandromorphic daphnids were randomly selected, sorted equally into 440 Hz, 432 Hz, and control groups, and high-frequency sounds were applied in aquatic conditions. During the 35-day trial period, the heartbeats, and egg numbers were counted whereas the survival rates were recorded. The results showed that the high-frequency sound exposure immediately showed its detrimental effect on heartbeats after a short adaptation period in the 440 Hz group and the average values decreased to half of the normal values. In the 432 Hz group, heartbeats first increased, and this level was maintained for a while, but then a significant decrease was observed. Regarding the egg numbers, an increasing trend was observed in the 432 Hz group until the 5th measurement period which resulted in a sharp decrease. In the 440 Hz group, lower values with almost no increase were recorded, except for the first measurement period, but no eggs were found in the 5th measurement. Interestingly, while no death was noted during the measurement periods, no living daphnia was observed in the 440 Hz group within 2 weeks during the non-measurement period. As a result, it was understood that *Daphnia* perceives the vibrations produced by high-frequency sound in the aquatic environment as a stressor and cannot cope with its detrimental effects after a certain time depending on intensity and duration. While the 440 Hz frequency noise caused infertility, the 432 Hz frequency noise resulted in lower egg numbers and heartbeats.

Keywords: *Daphnia magna*, noise pollution, 432 Hz, 440 Hz, reproduction

INTRODUCTION

The sounds produced by living things in the aquatic environment have a long frequency range (from 0.1 Hz to 200 kHz). They are produced by the mechanical hitting of teeth and plates, the movement of the body in the water, and the distribution of fluids or gases in the body through sound generating organs (Anonymous, 2021). The creatures living in the aquatic environment use sound to communicate due to courtship, habitat determination, feeding, hunting, and behavioral reproductive functions (de Vincenzi et al.,

2021; Coquereau et al., 2016). On the other hand, underwater noise pollution adversely affects the balance of marine living organisms (Bittencourt et al., 2014) which is not visible. The behavioral characteristics of creatures living in marine or freshwater ecosystems for basic life activities such as foraging, communication, and mating behavior are extremely sensitive to the effects of environmental noise. Therefore, scientific studies play important roles to understand the direction it affects aquatic organisms (Vakili et al., 2020).

Within the branchiopods, *Daphnia* belongs to Cladocera whose body is surrounded by a

non-calcified shell known as the shell. It has a double-wall, through which hemolymph flows and is part of the body cavity. The shell is largely made of chitin, a polysaccharide. *Daphnia* is a recognized model organism by the U.S. National Institutes of Health (Ebert, 2011). Several unique characteristics make it an attractive model organism for research into aging and regulation of life span, such as transparent body, short generation time, well-characterized methylome (Kvist et al., 2018). Moreover, *Daphnia* is a small crustacean that is inexpensive, easy to care for, and useful model organism for the cardiovascular function (Corotto et al., 2010; Kaas et al., 2009). It has a short life span, rapid maturation, and reproduction. Its physical transparency allows for simple observation of internal structures, including the heart (Teschner, 1995). *Daphnia* has an open circulatory system where a simple heart pumps the blood cells around. *Daphnia* is also easy to culture in the laboratory and can be maintained at high population densities in relatively small volumes of medium (Martins et al., 2007).

Sounds given externally to the aquatic environment are the noise sources produced by humans and these activities are defined as “Anthropogenic activities”. It is known that aquatic organisms are exposed to unnatural sounds from sonars, CHIRP, and multibeam devices (Vazzana et al., 2020), the noise of watercraft, dredging operations, marine constructions, oiling activities (Bittencourt et al., 2014), or extreme sounds created by explosions, air guns, and pile driving. These noise sources are known to adversely affect the fish, invertebrates, and even planktonic creatures living in the aquatic environment (Jones, 2019; Popper and Hawkins, 2019; Di Franco et al., 2020).

In the studies it was stated that the noise pollution detected in the Mediterranean is at the level of 190–230 dB. It was reported that in mammals, there are abnormal reactions in living things at 120 dB, temporary hearing loss around 160 dB, and definite hearing loss in noise above 180 dB (Anonymous, 2021).

In this context, the European Parliament and the Council of the European Union have also created a framework for community action in the field of marine environmental policy, and the 2008/56/EC Directive of the European Parliament and the Council (Marine Strategy Framework Directive) of 17 June 2008 also includes underwater noise pollution accepted as a kind of pollutant.

The present study was designed to determine the responses of *Daphnia magna* (water flea) exposed to 432 Hz and 440 Hz frequency sound, to assess the effects in an aquatic environment.

MATERIALS AND METHODS

Experimental crustaceans, rearing conditions, and sound exposure

This research was conducted in the Fisheries and Diseases Laboratory of the Faculty of Veterinary Medicine at Tekirdağ Namık Kemal University. Experiments were carried out with *Daphnia magna*, a planktonic crustacean belonging to the Branchiopoda cultured under laboratory conditions. The experiments were carried out in the laboratory, imitating the natural conditions in an aquarium environment using fresh water.

The experiment was conducted in a completely randomized design with 3 treatments and two replications. A total of 150 gynandromorphic daphnids were randomly selected, sorted equally into 440 Hz, 432 Hz, and control groups. They were placed into 6 aquariums each consisting of 25 *Daphnia* and were daily fed with *Daphnia* growth food (Inve O.range Start-S 100–200 μ) with an ingredient of 56% min raw protein, 13% raw fat, 1% raw cellulose, 1,2% max Ca, 1,3 max P and 10% ash. During the 35-day trial period, 50 daphniids were exposed to 432 Hz and 50 daphniids were exposed to 440 Hz sound frequencies for 1 h every day while the control group was not exposed to and sound. The media was changed every day. Survival was monitored daily and neonates were removed from the aquariums. The water temperature was kept at 25–26°C during the whole experiment.

As routine measurements pH, salinity, Total Dissolved Solids (TDS), conductivity, water (aquarium) temperature measurements were recorded with a waterproof ExStik® II pH/conductivity meter, EC500, “Extech” brand device, ambient temperature and humidity with a “Thermo HYGRO” device at intervals of 2 times a week. During the trial, biological filtration was applied by pipe filters separately in each experimental aquarium using a SunSun brand aerator (19 m³/h). The optimum growth conditions were achieved by applying a 30% water change in two-day intervals and keeping the nitrate level at an average of 10 ppm.

Measured parameters

Heart rate

In the measurement days, *Daphnia* was taken out of the aquarium by a tankard along with the same water. Each daphnia was removed from the tankard with a pipette and placed on a microscope slide and viewed through a microscope. Heart rate was recorded by counting the beats per minute using a light microscope, stopwatch, and slide at 7-day intervals.

Egg numbers

The egg numbers were recorded by counting the number of eggs at the top of the back, just behind the heart, in a dorsal brood pouch, which is the space between the carapace and the dorsal side of the trunk at 7-day intervals using a light microscope after the heartbeat measurements.

Survival rates/Mortalities

The survival rates were recorded at 7-day intervals until the end of the measurement periods.

Statistical analysis

The IBM SPSS Statistics 25.0 package program was used for statistical analysis. Heart rate and egg numbers were compared by the analysis of variance test, while the comparisons between groups were performed by the Tukey test. The comparison between two groups was made by Student's t-test. The results of mortalities after the measurement periods did not require statistical analysis.

RESULTS

Rearing conditions

The research was carried out under standard laboratory conditions and care was taken not to make any difference between the groups in terms of values inside and outside the aquarium (Table 1). The measurements were taken 2 times a week and it was observed that each average value of 10 measurements and the minimum and maximum values were close to each other.

Heart rate

The average heart rate values per minute were 339 beats for the control (range: 248-424), 336 beats for the 432 Hz (range: 204-436), and 284 beats for the 440 Hz (132-412) groups during the whole experiment. However, the effects of high-frequency sound on the parameters considered in the study were observed more clearly on a period basis (Table 2). There was a significant difference between the groups in terms of heartbeats in all periods except for the first measurement period ($p < 0.05$). While there was consistency in the control group in all measurement periods, variability was noticeable in the other experimental groups. In the 432 Hz group, an increase was observed in the first 3 measurement periods and a decreasing trend in the next 2 periods. In the 440 Hz group, an average similar to the other groups was observed in the first measurement period, but lower values were recorded in the 2nd, 3rd and 4th measurement periods whereas the lowest in the last measurement period with a significant decrease.

Table 1. Maximum, minimum and average value of environmental conditions in and outside the aquarium

Parameters	Groups	n	$\bar{x} \pm SD$	Min	Max
pH	Control	10	8.04 \pm 0.8	8.0	8.2
	432 Hz	10	8.16 \pm 0.8	8.0	8.2
	440 Hz	10	8.04 \pm 0.8	8.0	8.2
Salinity (mg/L)	Control	10	369.0 \pm 21.1	344	402
	432 Hz	10	354.8 \pm 8.1	346	368
	440 Hz	10	358.2 \pm 4.7	352	364
Total Dissolved Solids (TDS) (mg/L)	Control	10	588.2 \pm 26.9	552	626
	432 Hz	10	574.0 \pm 15.3	548	592
	440 Hz	10	578.6 \pm 2.8	574	582
Conductivity ($\mu S cm^{-1}$)	Control	10	708.0 \pm 67.5	601	796
	432 Hz	10	714.8 \pm 13.9	690	729
	440 Hz	10	719.0 \pm 4.6	712	725
Water temperature ($^{\circ}C$)	Control	10	26.6 \pm 0.5	26.1	27.2
	432 Hz	10	26.8 \pm 0.8	26.0	27.9
	440 Hz	10	26.8 \pm 0.6	25.7	27.5
Ambient temperature ($^{\circ}C$)	Control	10	27.7 \pm 0.8	27.0	29.1
Ambient humidity (%)	Control	10	61.8 \pm 2.8	59	66

Table 2. Comparison of heart rates of groups by periods

Periods	Groups	n	$\bar{x} \pm S_x$	p
1	Control	50	338.96 ± 5.45	0.954
	432 Hz	50	340.96 ± 5.18	
	440 Hz	50	338.88 ± 5.62	
2	Control	50	345.28 ± 5.93 ^b	0.000
	432 Hz	50	358.88 ± 4.12 ^b	
	440 Hz	50	295.60 ± 5.39 ^a	
3	Control	50	337.68 ± 4.91 ^b	0.000
	432 Hz	50	362.72 ± 5.46 ^c	
	440 Hz	50	309.36 ± 8.25 ^a	
4	Control	50	329.92 ± 3.81 ^b	0.000
	432 Hz	50	340.80 ± 4.22 ^b	
	440 Hz	50	295.36 ± 4.63 ^a	
5	Control	50	344.32 ± 4.28 ^c	0.000
	432 Hz	50	275.84 ± 6.68 ^b	
	440 Hz	50	178.80 ± 3.29 ^a	

p < 0.05; p: ANOVA; Different letters indicate significant difference among treatments by Tukey test.

In the 440 Hz group, the mean heart rate in the first 4 measurements was 310 beats per minute and declined significantly to 179 beats in the 5th measurement. This was the most dramatic result in terms of the average heart rates.

Egg numbers

The average egg number per daphnia during the whole experiment was 6 eggs for the control (range: 0-12), 6 eggs for the 432 Hz (range: 0-14), and 3 eggs for the 440 Hz (0-10) groups. Although these numerical values give the impression that there is a close variability to each other, the values based on each measurement period revealed the effect of high-frequency sounds on the number of eggs more clearly (Table 3).

There was a significant difference between the groups in terms of the egg numbers in all periods except for the first measurement period (p < 0.05). An increasing trend was observed in the 432 Hz group until the 5th measurement period which resulted in a sharp decrease. In the 440 Hz group, lower values with almost no increase were recorded, except for the first measurement period, but no eggs were found in the 5th measurement.

Mortalities

The experiment in which Daphnids were exposed to different frequency sounds lasted 35 days. When the number of eggs decreased to zero in the 440 Hz group, the sound applications as well as the heart and egg count

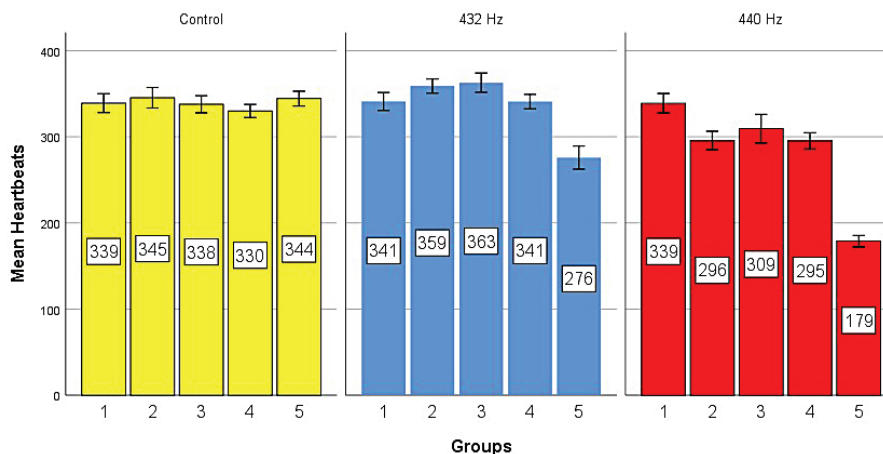


Figure 1. Mean heartbeats in groups by periods

Table 3. Comparison of egg numbers between groups by periods

Periods	Groups	n	$\bar{x} \pm S_x$	p
1	Control	50	3.36 ± 0.20	0.080
	432 Hz	50	3.90 ± 0.22	
	440 Hz	50	3.96 ± 0.20	
2	Control	50	4.64 ± 0.28 ^b	0.000
	432 Hz	50	4.78 ± 0.25 ^b	
	440 Hz	50	3.46 ± 0.21 ^a	
3	Control	50	6.98 ± 0.36 ^b	0.000
	432 Hz	50	7.56 ± 0.34 ^b	
	440 Hz	50	4.98 ± 0.33 ^a	
4	Control	50	7.10 ± 0.31 ^b	0.000
	432 Hz	50	8.38 ± 0.34 ^c	
	440 Hz	50	4.54 ± 0.27 ^a	
5	Control	50	8.44 ± 0.32 ^b	0.000*
	432 Hz	50	3.54 ± 0.22 ^a	
	440 Hz	50	0.00 ± 0.00	

p < 0.05; p: ANOVA; Different letters indicate significant difference among treatments by Tukey test.
 p < 0.05; *p: Student t-test; Different letters indicate significant difference between groups.

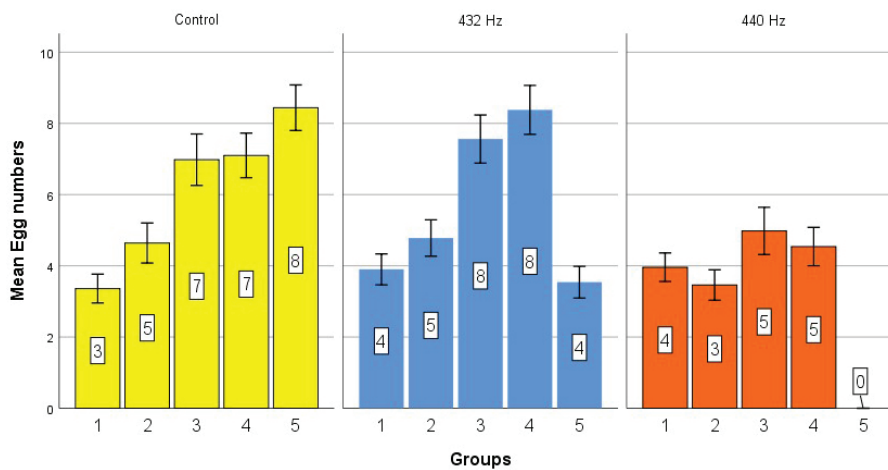


Figure 2. Mean egg numbers in groups by periods

measurements were terminated, but the experimental setup was kept the same for a while. While no death was noted during the measurement periods, no living daphnia was observed in the 440 Hz group in two weeks during the non-measurement period.

DISCUSSION

Although all aquatic vertebrates and invertebrates can hear sounds and are sensible to noise, fewer studies were published on the effects of noise pollution in marine invertebrates. In this context, this experiment was set up to test the responses of Daphnia exposed to high-frequency

noise under laboratory conditions, as an invertebrate and a model aquatic organism.

Temperature is an important factor determining the life cycle and fitness of all organisms including *D. magna*. When the water temperature rises, the metabolic rates of *D. magna* increase exponentially. They show a decrease in survival time and depending on the exposure length, they reduce their body sizes. A water temperature ranging from 23 to 26°C can be considered optimum, whereas at over 29°C, it is lethal for *D. magna* individuals (Mitchell and Lampert, 2000). In the current study, the water temperature was maintained at an average of 26.7°C with a range from 25.7 to 27.5°C with no adverse effect. Salinity and pH are other significant environmental

factors that negatively affect *D. magna* in terms of their reproduction, growth, survival, and energy allocation when occurring beyond the normal limits (Thabet et al., 2017). The salinity values ranged from 344 to 402 mg/L while the pH values were between 8 and 8.2 in this study within normal ranges. Since the level of total dissolved solids (TDS) in the freshwater must be less than 1,000 ppm (Anonymous-b, 2021), the range in this study from 552 to 626 is consistent with what is reported in the literature. Considering the optimum conductivity ranging from 600 to 1000 $\mu\text{S cm}^{-1}$ (Elizalde-Velazquez et al., 2020), the values in the present study between 601 and 796 $\mu\text{S cm}^{-1}$ are in accordance with the reference values.

Regarding the results of this experiment, a stable trend was observed in heart rate values in the control group in all measurement periods. However, it was noted that the reactions in heart rate against high-frequency sound differed in the 432 Hz and 440 Hz groups. In the 432 Hz group, the heartbeat rate per minute first increased from 341 beats to 359 beats, and then this level was tried to be maintained for a while, but then a significant decrease from 341 beats to 276 beats was observed. In the 440 Hz group, the heartbeat rate first decreased a certain amount from 339 beats to 296 beats, after staying at the same level for a while, a dramatic decrease from 295 beats to 179 beats was observed. From these changes, it was understood that in the 432 Hz group, the heartbeat increased with the effort to adapt to the sound and followed this way for a certain time, but after a while, even at low frequency, the sound application caused the heartbeats to decrease below normal. In the 440 Hz group, it was determined that the high sound frequency immediately showed its detrimental effect, and after a period of adaptation effort, it decreased to half of the normal values.

The frequency-dependent impacts of high-frequency noise on fertility showed that not only metabolic effects were seen in daphnia, but also the reproductive cycle was broken. The exposure to 440 Hz sound caused infertility with no eggs in the 5th measurement period and harmed the metabolism of *Daphnia*. Besides, it was revealed that 432 Hz sound application had negative effects on both heartbeat rate and egg number after a certain time. The sudden drop from an average of 8 eggs to 4 eggs was an obvious sign of that harmful effect. This was a surprising finding because generally 432 Hz sound is known as

a low-frequency sound and has positive effects on living things (Di Nasso et al., 2016; Exbrayat and Brun, 2019; Halbert et al., 2018). The values that were observed to be positive at the beginning were a reflection of the effort to cope with stress, and in the later stage, *Daphnia* could no longer cope with it. Together, the data in 432 and 440 Hz groups indicate that high-frequency sounds irritate aquatic organisms since they were perceived as vibrations no matter what frequency they were and caused stress in the aquatic environment. Therefore, outsourced noise disturbs animal behavior and physiology with negative consequences at the population level. Additionally, it was reported that continuous high-frequency noise impaired social interactions and communication (Butler and Maruska, 2020).

One of the most striking findings of the study was that the application of the 440 Hz sound, beyond the negative effect on the reproductive ability, caused the death of all *Daphnia* in the group. If the negative effect on reproduction is interpreted as an acute effect, the effect leading to death can be named as a chronic effect. Thus, it can be said that the organism's metabolic activities are affected step by step or that it reacts gradually to maintain its vital functions.

As a result, we hope that the results of this study will provide useful information about the effects of high-frequency noise on aquatic organisms.

CONCLUSIONS

Daphnia perceives the vibrations produced by high-frequency sound as a stressor and cannot cope with its detrimental effects after a certain time depending on intensity and duration. While the 440 Hz frequency noise caused infertility and heart rhythm disturbance, the 432 Hz frequency noise resulted in lower egg numbers and irregularity in heart rhythm. Therefore, as many fish and invertebrates play key ecological roles in marine life, more attention should be paid to noise pollution.

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