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Effects of Polystyrene Microplastics on Sound Production of Medaka Fish (*Oryzias latipes*)

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Microplastic pollution is increasingly recognized as a major global environmental issue. Studies have shown that microplastics (MPs) can affect various aspects of fish biology, including physiology, neurotoxicity, and behavior. To explore the potential of Japanese medaka (*Oryzias latipes*) as an indicator of MP pollution, we evaluated the effects of polystyrene microplastic (PS–MP) exposure on sound production. Test fish were assigned to one of three conditions: control, low concentration PS–MP exposure (0.01 mg/L), and high concentration PS–MP exposure (0.1 mg/L). Each fish underwent a sound production test on days 4 and 7. Exposure to high concentrations of PS–MP significantly lengthened the average sound inter-pulse interval, along with notable changes in other vocal trait parameters showing a reduced sound production activity upon exposure. These findings suggest that Japanese medaka sound production could serve as an effective biomonitoring tool for abnormal water conditions, including for detection of PS–MP and possibly other pollutants.

Key words: Microplastic, Japanese medaka, Sound production, Fish behavior

INTRODUCTION

Microplastic (MP) pollution has been gaining attention and concern from the scientific community in recent years. MPs been reported throughout Japanese freshwater systems, with concentrations closely linked to urbanization—particularly in densely populated areas with intense human activity. Their distribution patterns resemble those of other common pollutants, highlighting environmental degradation and underscoring the need for targeted monitoring and management in MP pollution hotspots (Imbulana *et al.*, 2023; Kataoka *et al.*, 2019). MPs are typically described as plastic particles with sizes ≤ 5 mm. Their small size makes them easily and rapidly consumed by aquatic species, posing potential threats to their survival. Toxicological studies suggest that MPs may cause harm through the leaching of their chemical additives, some of which act as endocrine-disrupting chemicals (EDCs), which may eventually be absorbed by organisms (Rios-Fuster *et al.*, 2022). These substances can interfere varying biological processes in aquatic as well as terrestrial organisms, impairing their mobility,

reproduction, neurological function and development (Cole *et al.*, 2011; Wang *et al.*, 2022; Zhang *et al.*, 2022).

Toxicity tests using fish can be traced back to as early as 1863 and have evolved considerably since then, leading to the development of numerous standardized testing criteria and techniques (Hunn, 1989). Behavioral alterations observed in fish are now widely utilized as tools for environmental risk assessment. Addressing the escalating plastic pollution, dependable techniques are needed to both identify the problem and measure its impact on living organisms.

Although visual-based recordings have been studied and used for toxicological assessments in recent years (Khalil *et al.*, 2017; Qiu *et al.*, 2017; Richendrfer *et al.*, 2012; Takai *et al.*, 2023; Tamura *et al.*, 2024), we aim to explore the feasibility of using vocal traits as an endpoint for MP detection for pollution monitoring efforts. In this study, we conducted experiments to investigate time-course alterations in the sound production activities of Japanese medaka (*Oryzias latipes*) upon polystyrene microplastic (PS–MP) exposure under controlled laboratory conditions. Recognized as a model organism for toxicology by the Organization for Economic Cooperation and Development (OECD, 2019), Japanese medaka is native to Japan and typically inhabits slow-flowing waterbodies such as rice paddies and ponds. It is well-suited for laboratory studies because of its short generation time, daily spawning, and adaptability to laboratory conditions (Wakamatsu and Ozato, 2003). Japanese medaka is also considered a sonic fish that communicates acoustically on a regular basis. In their natural habitat where there is turbidity, sound production may be useful as part of their communication system, as visual or olfactory sensors can become less reliable. Sound production in fish is known to occur during courtship,

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spawning, agonistic behavior, competitive feeding, and in situations where they are disturbed (Fine and Parmentier, 2015).

Previous studies have shown alterations in sound production of Japanese medaka in response to the heavy-metal compound copper sulfate, the insecticide aldicarb, as well as the organophosphorus pesticide chlorpyrifos (Kang *et al.*, 2017; Zhuo *et al.*, 2024). Expanding on this concept, it might be possible to develop a method of water pollution detection using Japanese medaka sound production, which would be applicable on-site where turbidity can limit visual analyses. Our study spanned a one-week exposure period, where vocal traits were recorded twice, on the fourth and last days of exposure. The study aimed to evaluate the sensitivity of responses to MP exposure and explore the potential of using Japanese medaka as a bioindicator for MP pollution.

MATERIALS AND METHODS

Polystyrene microplastics

The polystyrene microplastics (PS-MP) used were 2- μm green fluorescence-labeled microspheres (excitation peak, 488 nm; emission peak, 518 nm) purchased from BaseLine ChromTech Research Center (Tianjin, China).

Test fish

The Japanese medaka were purchased from Chemicals Evaluation and Research Institute, Japan (CERI) and acclimatized to laboratory conditions before the exposure tests.

At the laboratory, Japanese medaka were kept in an aquarium containing dechlorinated water with a salinity of 0.01%. The water was changed once a week. Test fish were fed brine shrimp *Artemia* nauplii twice a day: in the morning at 09:00 and in the evening at 18:00. They were conditioned under a photoperiod of 14-h:10-h light:dark with water temperature maintained at 24.1 ± 1 °C.

PS-MP exposure test

The test exposure lasted for 7 days, where each group of 20 fish (0.34 ± 0.064 g; 0.32 ± 0.026 cm) was first introduced into a 20-L glass tank filled with 18 L test solution (temperature, 23.7 ± 1 °C), mixed through aeration (Fig. 1A). Test fish in the control group were placed in a tank with no added PS-MP. The low concentration group was in water with 0.01 mg/L PS-MP and the high concentration group in water with 0.1 mg/L PS-MP. Water in the tanks was changed every 48 hours during the exposure period. Test fish were fed with the powdered artificial diet Otohime B2 (Marubeni Nisshin Feed Co., Ltd., Japan) twice a day at 0900 and 1800 local time. They were kept in a room with a 14-h:10-h

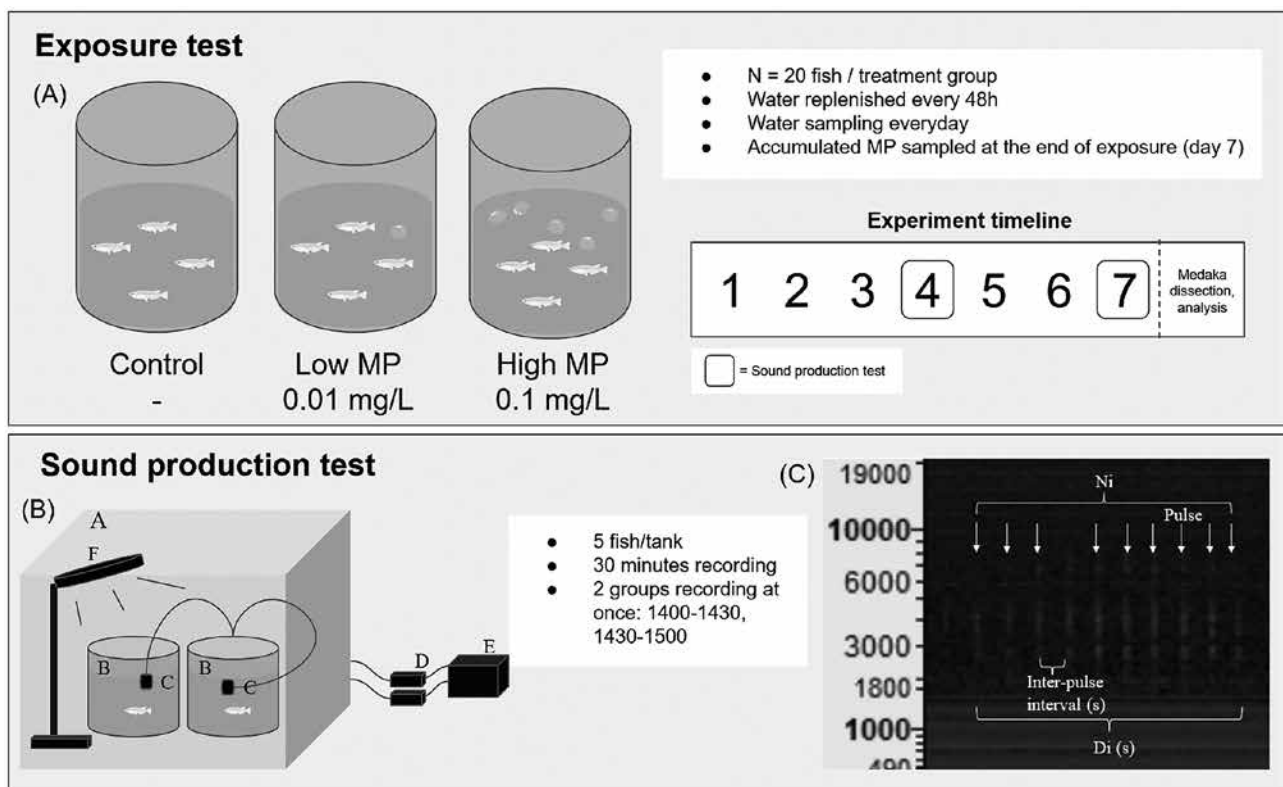


Fig. 1. Experiment design.

(A) Exposure test setup. (B) Illustration of the experimental setup for sound production analysis; soundproof box (a) contained two experimental containers (b), each with a hydrophone (c) connected to an amplifier (d) and converter (e), with artificial lighting (f). (C) An example of a spectrogram of a pulse group from a control group recording, where sound frequency (Hz, y -axis) is plotted against recording time (x -axis).

light:dark cycle.

Time-course analysis of sound production

We made audio recordings on days 4 and 7 to analyze sound production by the fish. Each exposure group was separated into four 1-L containers (Iwaki Co., Ltd., Japan) containing 1 L of water and acclimatized for at least half an hour. Each tank contained 5 fish, with both males and females mixed, to create conditions conducive to acoustic communication in a social setting and enable courtship activities. Two groups were recorded simultaneously. Each recording session lasted for 30 minutes. The 1-L tanks were placed inside a soundproof box with artificial lighting to simulate daytime (Fig. 1B). The light, along with a hydrophone (model AS-1; Aquarian Audio & Scientific, Anacortes, WA, USA), were connected to power through a cable opening in the box. The hydrophone was fixed such that it was immersed inside the water from above. The recording apparatus connected to the hydrophone included a hydrophone amplifier (model PA-4; Aquarian Audio & Scientific), and an audio converter (model UAC-2; ZOOM, Tokyo, Japan), which was connected to a laptop that stored the audio recordings. Once the recordings for the first two tanks were finished, the remaining two tanks underwent the same recording procedure.

The recordings were analyzed using the software Audacity (<https://www.audacityteam.org/>) to check the sound behavior of the fish. According to Kang *et al.* (2017), Japanese medaka produces sounds in sequences of pulses with a peak frequency range of 0.5–3.5 kHz and a constant average inter-pulse interval. Following this guide, we examined the spectrogram of each recording by identifying the pulses and the corresponding times.

To examine the sound patterns, we used the method of Kang *et al.* (2017) for determining the average inter-pulse interval (iPI_{ave}). From the data collected, we determined various measurements, including the number of pulses (NP), number of pulse groups (NPG), number of pulses (N_i) in each pulse group and the duration (D_i) of each pulse group (Fig. 1C).

Recordings were done at the same time for each group and day to avoid differences in communication activity that occur at different times of the day. Specifically, fish were recorded at 1400–1430 and 1430–1500 (with the exception of the high PS-MP concentration group on day 4, which was recorded at 1430–1500 and 1500–1530 because of unexpected instrument error).

Quantification of PS-MP in water and in Japanese medaka intestines

Throughout the 7 days of PS-MP exposure, water samples were collected from each tank every day to measure and monitor any changes in the amount of PS-MP available in the water. On days when there were water changes (days 2, 4, and 6), water samples were taken before and after the change. Water was collected into a glass beaker and kept for filtration.

Each sample was filtered through a 47-mm-diam-

eter glass microfiber filter (Whatman GF/C; Cytiva, Tokyo, Japan) using a glass filtration apparatus connected to vacuum suction. The PS-MP particles remained on the filter. The filter was then observed under a fluorescence microscope, and pictures were taken at five random spots to estimate the number of PS-MP particles using image processing software (ImageJ; Schneider *et al.*, 2012).

To determine the number of PS-MP particles in the intestines of test fish, the intestines were first dissolved in H_2O_2 and filtered through a 25-mm-diameter microfiber filter (Whatman GF/F) using a glass filtration apparatus. The subsequent procedures were similar to those for quantifying PS-MP in water. We had previously established a standard curve for both the area of PS-MP in water and in test fish intestines as detected by ImageJ against the corresponding amount of PS-MP utilized in the samples, which was used to obtain the PS-MP measurements in this study. The measured concentration was then adjusted accordingly: by the sample volumes for the water samples (calculated as mg/L) or by the body weights of test fish for the intestine samples (calculated as PS-MP/g-weight).

Statistical analysis

To evaluate each sound production parameter, we tested for statistical differences between groups using the R packages “multcomp” and “nparcomp” (Hothorn *et al.*, 2008; Konietschke *et al.*, 2015). Prior to analysis, data normality was assessed with the Shapiro-Wilk test, and variance homogeneity was evaluated using Bartlett’s test. When the assumption of equal variances was met, we applied one-way ANOVA or Tukey’s test for group comparisons. For non-parametric analysis, Steel’s test was employed.

Furthermore, we used the “MuMIn” package (ver. 1.48.11) in R to perform a generalized linear model (GLM) analysis aimed at identifying the best-fitting model to examine the relationship between PS-MP exposure concentration and medaka vocal traits. Given the presence of multiple explanatory variables and the need to pinpoint factors influencing the number of pulse groups (NPG), GLM, which has been applied in ecological and risk assessment studies (Kerr and Meador, 1996; Smith and Warren, 2019), was selected for this analysis. In addition to exposure concentration, we included the sequence of recording time (categorized as either the first or second pair recorded) and test temperature as explanatory variables to evaluate their influence on NPG, the response variable.

Ethics

All experiments were performed according to the Animal Experiment Regulations of Kyushu University.

RESULTS

PS-MP concentrations in test water

Spherical PS-MP particles of 2- μ m diameter were identified and confirmed through its fluorescence under

the microscope. There was a constant decreasing trend of PS–MP concentration in the test water for the exposure groups before an increase resulting from the water changes. In the sections that follow, we assumed the following nominal concentrations for the exposure tests: 0.01 mg/L and 0.1 mg/L for low and high PS–MP concentrations, respectively.

Accumulated PS–MP in Japanese medaka intestines

Our measurements of PS–MP in the intestines of test fish reflect the total uptake of PS–MP in Japanese medaka over the 7–day exposure period, the number of PS–MP accumulated increased with the concentration of PS–MP exposed in the water solution (Fig. 2A).

Effects of PS–MPs on Japanese medaka sound production

The average inter–pulse interval was shorter in the low concentration PS–MP exposure group but significantly longer in the high concentration PS–MP exposure group compared to the control group. The means of the average inter–pulse intervals for the control and low concentration exposure groups were 0.20 s and 0.19 s, respectively, whereas that for the high concentration exposure group reached 0.25 s. The average inter–pulse interval for the high concentration group reached to over 0.30 s. Tukey’s test revealed significant differences between the control and high–concentration PS–MP groups ($P < 0.05$) and between the low–concentration and high concentration PS–MP groups ($P < 0.01$).

Overall, there was an increase in the average inter–pulse interval from day 4 to day 7. However, because of missing of data for the control group on day 4, those recordings were excluded from the statistical tests. There were statistically significant differences between day 4 and day 7 in the low–concentration groups, as well

as on day 4 between the two exposure groups ($P < 0.01$).

Looking at other measured parameters related to sound production, we found alteration of sound production activity at day 7. One recording from each of the exposure group had pulses but no pulse groups (gaps between pulses are not close enough to be considered as pulse groups), making it impossible to calculate the average inter–pulse interval.

Generalized linear model for sensitivity of sound production parameter to PS–MP exposure

We selected the number of pulse groups (NPG) (Fig. 2B) recorded from each test as the vocal trait representing Japanese medaka sound production activity. Considering the potential bias that may form due to incomplete data from day 4 recordings of the control group, we focused on day 7 data to conduct GLM regression with Poisson distribution. Among the different variable combinations tested, Model 1 had the lowest Akaike’s information criterion (AIC) score, thus identifying it as the most suitable for evaluating changes in NPG in response to PS–MP exposure (Table 1, Equation no. 1). The equation derived from this model is as follows.

$$\log Y_{NPG} = -0.0747 \times X_{\text{Exposure conc.}} - 0.202 \times X_{\text{Recording time}} + 3.45$$

DISCUSSION

A seven–day exposure to PS–MP resulted in the accumulation of microplastic particles in the intestines of Japanese medaka. The ingested PS–MP may have contributed to physiological disruptions, consistent with earlier studies reporting behavioral alterations. In our sound production tests, we detected changes in vocal traits following PS–MP exposure, with significant differ-

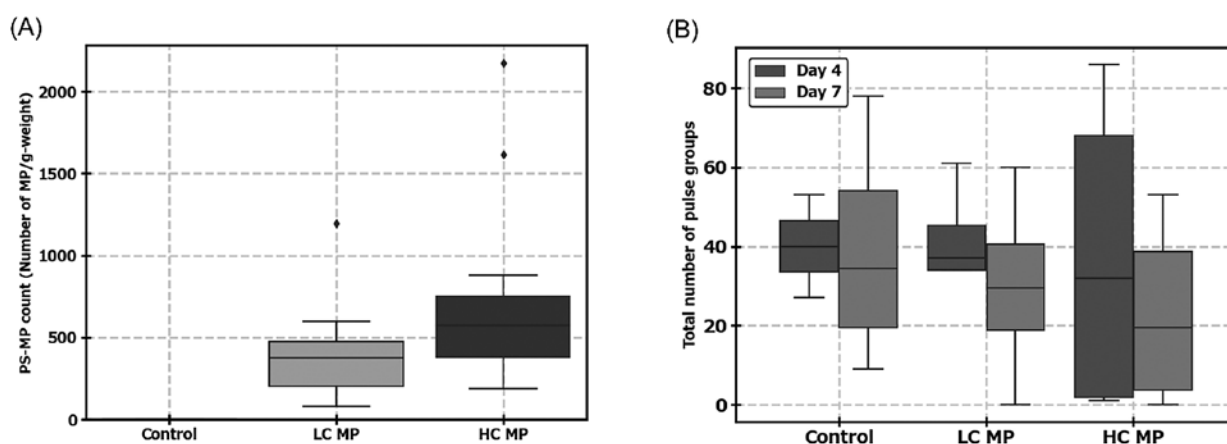


Fig. 2. Results of PS–MP exposure test.

(A) Box plot of PS–MP counts in test fish intestines. Normalized to body weight, each box plot represents the distribution of PS–MP counts, where the horizontal line inside the box indicates the median, the box bottom and top represent the first and third quartiles, respectively, and the whiskers extend to the minimum and maximum values. Plotted points outside the whiskers denote outliers. The labels in the x-axis represents PS–MP concentrations in the exposure test: Control, 0 mg/L; LC MP, low–concentration PS–MP, 0.01 mg/L; HC MP, high–concentration PS–MP, 0.1 mg/L. (B) Variations on the total number of pulse groups from sound production test of days 4 and 7.

Table 1. Result of GLM analysis to assess factors affecting NPG using different combinations of explanatory variables.

Equation	Exposure concentration	Recording time	Temperature	Intercept	AIC
1	-0.0747	-0.202	–	3.45	313.0
2	-0.0541	-0.202	-0.203	8.26	313.2
3	-0.0748	–	–	3.15	314.7
4	-0.0541	–	-0.203	7.96	314.9
5	–	-0.202	-0.402	13.1	316.2
6	–	–	-0.402	12.8	318.0
7	–	-0.202	–	3.72	326.0
8	–	–	–	3.42	327.8

“Exposure concentration” refers to the PS–MP concentration (log₁₀–transformed concentrations) used in the exposure test. “Recording time” refers to the order of sound production test recording. “Temperature” refers to the water temperature during the test. Explanatory variables underlined in the table were statistically significant ($P < 0.05$) within the model. AIC; Akaike’s information criterion.

ences in the average inter–pulse interval observed as early as day 4.

The average inter–pulse interval was significantly longer in the group exposed to a high concentration of PS–MP compared to the control group, which aligns with the changes observed in a previous study of Japanese medaka exposed to aldicarb (Kang *et al.*, 2017). The PS–MP concentration in our low concentration group might not have been sufficient to produce noticeable effects on Japanese medaka sound production. Alternatively, changes in sound production may vary with different concentrations of PS–MP.

Kang *et al.* (2017) also reported a reduction and eventual cessation of sound production upon copper sulfate exposure. In our study, there were instances where no sound pulses were detected from the exposure groups, particularly on the last day of exposure. These instances of lack of pulse groups suggest a longer D_i , which may point to a reduction in sound production potentially caused by PS–MP exposure. Additionally, the mean values for the number of pulses and pulse groups showed a decreasing trend across the exposure groups from day 4 to day 7, supporting the increasing trend in the inter–pulse interval. This indicates a reduction in sound production activity as the exposure duration increased.

From the GLM analysis, we derived a model (Equation 1) indicating that increased PS–MP exposure levels are associated with a reduction in a vocal trait parameter. The top three models ranked by AIC scoring consistently identified exposure concentration as a significant factor influencing NPG, with Model 1 showing significance at $p < 0.05$. All models showed negative coefficients for exposure concentration, suggesting that higher PS–MP exposure concentration led to a decrease in the pulse groups made by test fish during sound production. These results suggest that PS–MP exposure may impair physiological and behavioral functions related to sound production as reflected in the NPG.

Although the precise mechanism or reason for their sound production has yet to be fully understood, abnor-

mal vocal patterns and potentially altered communication activity—crucial for reproduction, social interactions, and other behaviors—raise concerns about decreased survivability and ecological continuity as essential functions are disrupted by PS–MP exposure. Tamura *et al.* (2024) reported alterations in Japanese medaka shoaling behavior upon PS–MP exposure, while our study found a reduced sound production activity using the same toxicant. This suggests a possible link between these two behavioral changes. Given that sound production likely plays a role in communication, a decrease in vocal activity could negatively impact social behaviors like shoaling, which PS–MP exposure also appears to disrupt. However, further research is needed to clarify the relationship between these behavioral effects.

Additionally, MP exposure has been reported to influence neurotransmitter pathways (Ghosh, 2024), potentially impairing muscle function. If muscles involved in auditory–related processes are affected, this could help explain the reduction in sound production observed.

Our study highlights the potential for using acoustic trait changes in Japanese medaka as an effective indicator for monitoring MP pollution. While our study included exposure conditions for multiple days, our findings suggest that behavioral testing could be integrated into on–site monitoring systems for more regular and automated detection of abnormal water conditions. This approach could offer a more time– and labor–efficient alternative to traditional field sampling methods. Building on this idea, further studies are needed to evaluate the feasibility of using behavioral testing in real aquatic environments by examining the effects at longer exposure times—investigating whether the effects would become more severe or whether there is possible recovery, exposure concentrations, as well as a variety of natural conditions and assessing the appropriate technologies or instruments for implementation. Additionally, by establishing that Japanese medaka exhibit measurable changes in sound production in response to MP expo-

sure on top of past studies on heavy metal and pesticide exposures, we highlight the species' acoustic traits as a sensitive parameter for detecting water quality changes, which may also be applicable for monitoring other environmental contaminants.

This study focused on the effects of exposure to 2- μ m PS-MP; however, it remains unclear whether similar effects would occur with other polymers or different particle sizes. Hence, there is a need to clarify the effects of MPs on Japanese medaka incorporating results from previous studies. A meta-analysis of MP exposure effects on Japanese medaka could help consolidate existing knowledge and guide future studies. This would enable the design of more targeted experiments, ultimately contributing to a clearer understanding of MP impacts.

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AUTHOR CONTRIBUTIONS

F. R. Zahra: Conceptualization, Investigation, Visualization, Writing – Original Draft Preparation; K. Komatsu: Investigation; Y. Takai: Conceptualization, Investigation, Supervision, Writing – Review & Editing; Y. Oshima and Y. Shimasaki: Supervision; I. J. Kang: Conceptualization, Supervision, Writing – Review & Editing.

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