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Short communication

Exposure of bay scallop *Argopecten irradians* to micro-polystyrene: Bioaccumulation and toxicity

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ABSTRACT

Marine microplastic pollution poses a threat to aquatic organisms, including bivalves. In this study, we investigated the accumulation of microplastics and their elicited antioxidant stress response in the bay scallop *Argopecten irradians*. Scallops were exposed to 1 μm diameter micro-polystyrene (MP) beads at 10, 100, and 1000 beads/mL concentrations for a 7 day period. Bead presence in the digestive diverticula and defense responses in the digestive diverticula and hemolymph were measured at 1, 3, 5, and 7 days. The activity and expression of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) and H_2O_2 in the digestive diverticula and/or hemolymph of scallops increased with microplastic concentration and exposure duration. These results suggest that microplastics can accumulate in the digestive diverticula of *A. irradians*, and that exposure to microplastics induces oxidative stress in bivalves. It is likely that exposure to high concentrations of micro- or nano-sized plastic particles could potentially have adverse effects in bivalves.

1. Introduction

The demand for plastic products has increased with human population growth, and annual production has reached > 300 million tons per year (Andrady, 2017). The resulting plastic debris is broken down into micro- or nanometer-sized fragments by physical and/or biological decomposition and UV radiation (Browne et al., 2008; Barnes et al., 2009; Canesi et al., 2015). Because microplastics smaller than 5 mm are buoyant, persistent, and easily distributed by currents, they are now present in every marine ecosystem (Lusher et al., 2013). They are highly likely to have harmful effects on marine ecosystems around the world. Ingested microplastics can accumulate in marine organisms (Browne et al., 2008), and can be transmitted up the trophic levels, posing greater threats to predatory taxa (Mattsson et al., 2014; Torre et al., 2014).

Microplastic accumulation in aquatic organisms adversely affects a number of physiological processes, including feeding, growth, immunity, and neurological function (Lee et al., 2013; Besseling et al., 2014). In lugworms and mussels, microplastics cause the production of reactive oxygen species (ROS) and oxidative stress (Browne et al., 2013; Avio et al., 2015). Small-bodied organisms may consume microplastics < 20 μm in size and may be able to discharge them (Lee et al., 2013; Wright et al., 2013).

Scallops have a high rate of toxin accumulation due to a low metabolic rate (Hannam et al., 2009). When exposed to toxic substances, various defense mechanisms are activated. Responses to toxic stress include the production of ROS, which migrate to hemolymph and peroxidize lipids in the tissues (Gao et al., 2008). Shellfish protect their tissues from oxidative damage by detoxifying ROS using antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Frantzen et al., 2016).

The bay scallop *Argopecten irradians* is a commercially important shellfish in the Atlantic and in Korea and China, and readily accumulates toxic substances in its tissues (Heffernan et al., 1988; Oh et al., 2003). Polystyrene is one of the most common types of marine microplastics and is widely used as a model for microplastic absorption and biological effects in various organisms (Farrell and Nelson, 2013; Besseling et al., 2014). Studies of bivalves (Mathalon and Hill, 2014; Davidson and Dudas, 2016; Murphy, 2018) have confirmed the accumulation of microplastics in scallops around the world. However, few studies have simultaneously confirmed changes in accumulation and antioxidant expression from exposure to microplastics at various concentrations.

We aimed to investigate the presence and mode of action of micro-polystyrene (MP) in the digestive gland of *A. irradians* and assess the potential ecotoxicological risk of this contaminant to this species by identifying changes in the expression of oxidative stress parameters.

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2. Materials and methods

2.1. Ethics statement

All experiments were carried out in strict accordance with the guidelines and ethical principles of the Experimental Animal Welfare Ethics Committee of Korea.

2.2. Experimental animals

A. irradians scallops with a shell length of 57 ± 11 mm were obtained from a commercial market in Goseong, Korea. They were kept for one week prior to the experiment in four 400 L circulation tanks containing filtered and aerated seawater maintained at 17 ± 2 °C and 31 ppt (salinity) using an automatic temperature regulation system (JS-WBP-170RP; Johnsam Co., Buchoen, Korea). Half the total volume of water in the tanks was changed daily.

2.3. Micro-polystyrene

Two types of 1 μm MP beads were obtained from Invitrogen (Carlsbad, California, USA). Fluorescent MP beads (FMP; Fluospheres™ Polystyrene Microspheres; F13080) were used for uptake and accumulation observations, and virgin MP beads (CML Latex Beads; C37483) were used for toxicity tests. Both types of microplastics used in the experiment (fluorescent or virgin) were made from polystyrene. We used concentrations ranging from 10 to 1000 beads/mL. This value is below the concentration used in previous laboratory exposure experiments (Browne et al., 2008; Avio et al., 2015; Sussarellu et al., 2016), although higher than environmentally relevant concentrations of microplastics found in seawater in highly contaminated regions, ~0.5 particles/mL in South Korea (Song et al., 2014).

2.4. Fluorescent micro-polystyrene accumulation in digestive diverticula

A. irradians were starved for 24 h and randomly divided into control and experimental groups into each tank filled with 80-L of filtered and aerated seawater. Each group consisted of two sub-groups of 20 scallops (20 × 2 = 40 per group for repeat experiments). Four experimental conditions were set up in replicate containing FMP beads suspended at concentrations of 0, 10, 100, and 1000 beads/mL, and hereinafter referred to as Cont., FMP 10, FMP 100, and FMP 1000. Five scallops from each replicate treatment group were sampled randomly after 1, 3, 5, and 7 days. Digestive diverticula tissues from sampled scallops were extracted and fixed in buffered formalin solution and dehydrated in increasing concentrations of ethanol solution, clarified in xylene, and embedded in paraffin. Sections (6 μm thick) were selected and stained with hematoxylin and eosin for observation under a fluorescence microscope (excitation filter 465–495 nm; Eclipse Ci, Nikon, Japan). Images were captured using a digital camera (Eclipse Ci, Nikon, Japan) to determine the presence of microplastics. The background fluorescence of the tissues of the control was detected and subtracted from that of FMP treatment samples. In order to observe the presence of FMP in tissues, one bright-field image was acquired by microscopy first and then a dark-field image of the same slide was acquired by fluorescence microscopy. FMP accumulation measurements were made using image analysis software (Image Pro Plus, v.4.5, Media Cybernetics, Inc., USA).

2.5. Micro-polystyrene toxicity

A second set of acclimated scallops were randomly divided into control and experimental groups into tanks filled with 80-L of filtered and aerated seawater. Each group consisted of two sub-groups of 20 scallops (20 × 2 = 40 per group for repeat experiments). Four experimental conditions were set up in replicate containing virgin MP suspended at concentrations of 0, 10, 100, and 1000 beads/mL, and therefore referred to as Cont., MP 10, MP 100, and MP 1000. Five scallops from each replicate treatment group were randomly selected after 1, 3, 5, and 7 days. One milliliter of hemolymph was collected from the adductor muscle using a 1 mL syringe within 2 min of removing scallops from the tank, after which digestive diverticula tissues were collected. The hemolymph was separated by centrifugation at 4 °C and 750 ×*g* for 3 min. All samples were stored at −80 °C prior to analysis.

2.6. Expression of SOD and CAT

Total RNA was extracted from the digestive diverticula using TRI reagent® (Molecular Research Center, Inc., Ohio, USA) according to the manufacturer's instructions. RNA (2 μg) was reverse-transcribed in a volume of 20 μL, using an oligo-d$(T)_{15}$ anchor and M-MLV reverse transcriptase (Promega, Madison, WI, USA). Synthesized complementary DNA (cDNA) was stored at −20 °C. Relative expression of SOD (GenBank accession no. **EU137676**), CAT (GenBank accession no. **GQ265925**), and ribosomal protein S18 (RPS18; GenBank accession no. **AF526232**) were measured using qPCR following primers: SOD forward primer, 5′- TAG GGA TTT TGG CTC GTT TG -3′; MnSOD reverse primer, 5′- GTA GGC ATG CTC CCA AAC AT -3′; CAT forward primer, 5′- TAC TGC AAG GCC AAG CTT TT -3′; CAT reverse primer, 5′- TTG GGA AAA ATA TGG GGT CA -3′; RPS18 forward primer, 5′- GTC TGC AAG AAG GCT GAT GT -3′; RPS18 reverse primer, 5′- GGG TTG GAC ATG ATT GTG AT -3′. PCR amplification was conducted with a Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad) and iQ™ SYBR Green Supermix (Bio-Rad) using 0.5 μL cDNA, 0.26 μM forward and reverse primers, 0.2 mM dNTP, 10 μL SYBR Green, and Taq polymerase in buffer (10 mM Tris–HCl at pH 9.0, 50 mM KCl, 1.4 mM $MgCl_2$, and 20 nM fluorescein) to a total volume of 25 μL. Amplification was carried out at 95 °C for 5 min, followed by 40 cycles of 95 °C for 20 s and 55 °C for 20 s. As internal controls, experiments were duplicated with RPS18, and all data were expressed relative to the corresponding RPS18 threshold cycle using the $2^{-\Delta\Delta Ct}$ method.

2.7. SOD, CAT, and H_2O_2 activities

SOD, CAT, and H_2O_2 activities in hemolymph were respectively measured using the BM-SOD200, BM-CAT400, and BO-PER500 assay kits (BIOMAX Co., Ltd., Korea) according to the manufacturers' instructions. In the SOD assay, oxidation of xanthine was monitored by measuring absorbance at 450 nm. One unit of SOD activity was defined as 50% inhibition of the oxidation process (U/mL hemolymph). One unit of CAT activity was defined as 50% H_2O_2 consumption in 1 min at pH 7.0 (U/mL hemolymph).

2.8. Statistical analysis

All data were analyzed using SPSS version 25.0 (IMB SPSS Inc., USA). For all the parameters analyzed, exposure to different concentrations and samples at different times of exposure were compared using two-way ANOVA. ANOVA assumptions (Levene's test for homogeneity of variances and the Kolmogorov-Smirnov test for data normality) were checked. Where significance was indicated, the Tukey post hoc test for multiple comparisons was used. If necessary, data were mathematically (log function) transformed to meet the ANOVA assumptions. The significance level adopted was 95% ($P < 0.05$). Values are expressed as the mean ± standard deviation (SD).

3. Results

3.1. Accumulation of micro-polystyrene

FMP accumulation occurred in the digestive diverticula of scallops at all tested microplastics concentrations and occurred faster under

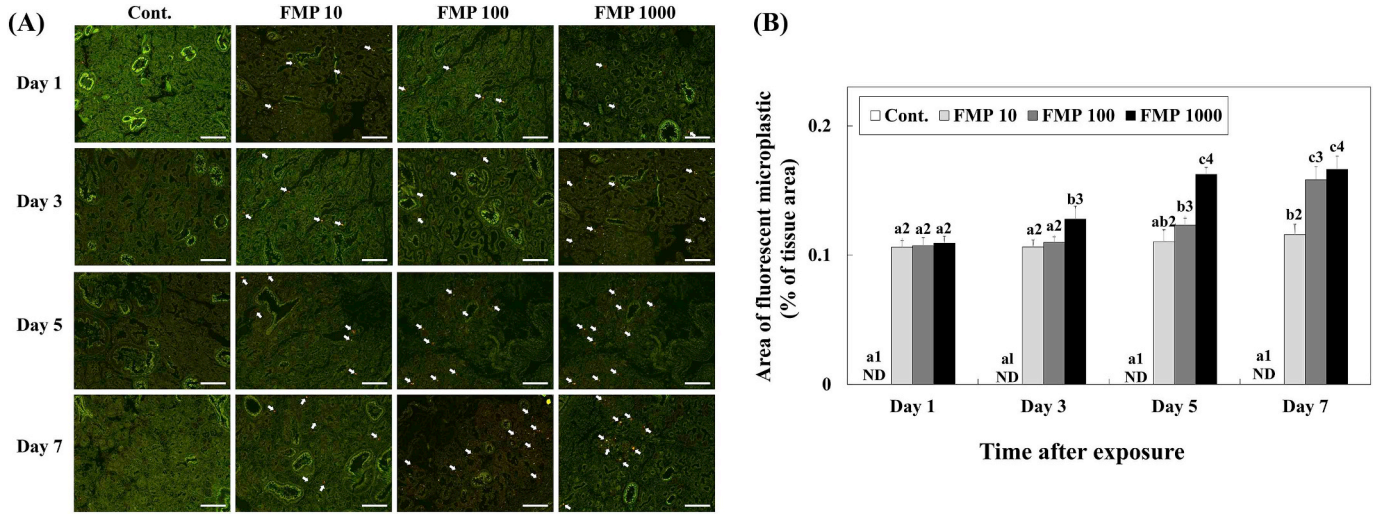

Fig. 1. (A) H&E stained tissue sections of the digestive diverticula of *Argopecten irradians* exposed to fluorescent micro-polystyrene (FMP) for 7 days. White arrows indicate microplastic accumulation. Scale bar = 200 μm. (B) Quantification of H&E images of the digestive diverticula after exposure to FMP for 7 days. Changes in areas of FMP relative to tissue area (%). Different letters indicate significant differences among scallops exposed to the same microplastic concentration for different durations ($P < 0.05$). Different numbers indicate significant differences among scallops exposed to different microplastic concentrations for the same duration ($P < 0.05$). ND = not detected.

greater concentrations (Fig. 1). In groups, the accumulation treatment continued until the end of the experiment on day 7. Accumulation was highest in the group treated with 1000 beads/mL on day 7 (0.17 ± 0.01%, $P < 0.05$).

3.2. Antioxidant enzyme expression and activity

In the group treated with MP at 10 beads/mL, SOD expression (0.27 ± 0.02, $P < 0.05$) and activity (0.39 ± 0.02 U/mL, $P < 0.05$) increased significantly from the baseline beginning on day 3 and continued to increase over time (Fig. 2A and B). In groups treated with 100 and 1000 beads/mL, SOD expression and activity increased significantly beginning on day 1 ($P < 0.05$), and continued to increase until the end of the experiment. Both measures were significantly higher in the 1000 beads/mL group compared with any other group (at day 7, mRNA; 0.60 ± 0.04, activity; 0.99 ± 0.07 U/mL, $P < 0.05$). There were significant interactive effects between factors (SOD expression; $F_{3,\ 144} = 480.934$, $P < 0.01$, activity; $F_{3,\ 144} = 322.024$, $P < 0.05$). On day 1, CAT expression in the groups treated with 100 (0.38 ± 0.02) and 1000 (0.38 ± 0.04) beads/mL increased significantly ($P < 0.05$, Fig. 2C). CAT activity increased in all treated groups compared to controls from the baseline beginning on day 3 (Fig. 2D). Expression and activity were significantly greater in the group treated with 1000 beads/mL compared with all other treated groups. CAT expression and activity increased significantly with microplastic concentration and duration, with the highest values observed on day 7 in the 1000 beads/mL treatment (mRNA; 0.85 ± 0.04, activity; 1558.46 ± 77.92 mU/mL, $P < 0.05$). There were significant interactive effects between factors (CAT expression; $F_{3,\ 144} = 290.211$, $P < 0.05$, activity; $F_{3,\ 144} = 180.788$, $P < 0.05$).

3.3. H_2O_2 activity

The H_2O_2 concentration in the hemolymph rose significantly with increasing microplastic concentrations (Fig. 2E) and was significantly higher in the group treated with 1000 beads/mL compared with the other groups ($P < 0.05$). All treated groups showed greater H_2O_2 concentration than the control at each time point, with the highest concentration observed on day 7 in the group treated with 1000 beads/mL (56.26 ± 2.8 μM, $P < 0.05$). There were significant interactive effects between factors ($F_{3,\ 144} = 388.490$, $P < 0.01$).

4. Discussion

In our study, microplastics in the digestive diverticula accumulated in line with experimental concentrations and duration. Scallops most likely absorbed microplastics via the gills (Browne et al., 2008). When blue mussels *Mytilus edulis* were exposed to 3.0 and 9.6 μm microplastics, the particles were transferred to the circulatory system within 3 days, then remained in the tissues for at least 48 days (Browne et al., 2008). Eastern oysters *Crassostrea virginica* likewise showed polystyrene absorption via the gills and accumulation in the digestive diverticula, with a greater accumulation of 50 nm particles than 3 μm particles. The absorption and accumulation increased with concentration and exposure duration (Gaspar et al., 2018). This suggests that even if scallops repeat the intake and egestion of microplastics through selective feeding (Ward et al., 2019), microplastics of 1 μm or less will begin to accumulate in the tissues within a day. This may lead to microplastic ingestion by predators higher up the food chain.

The expression and activity of the antioxidant enzymes SOD and CAT and H_2O_2 increased as our experiment progressed. This indicates that microplastics have toxic effects on *A. irradians*, with antioxidant enzymes required to eliminate ROS generated as part of the toxicity process (Gao et al., 2008). When *Scrobicularia plana* bivalves were exposed to 20 μm microplastics for 21 days, microplastics become detectable in the hemolymph, and the activities of antioxidant enzymes and H_2O_2 increased over time (Ribeiro et al., 2017). Additionally, lipid peroxide concentrations increased in the digestive gland. This has also been observed in *M. edulis* (Magara et al., 2018) and zebrafish (Lu et al., 2016), with antioxidant enzyme expression increasing with duration and microplastic concentration of either polystyrene or polyethylene. *Tigriopus japonicus*, a grazing copepod, also showed increased expression of antioxidant enzymes over time with exposure to microplastics (Choi et al., 2020). This suggests that antioxidant enzymes are overexpressed due to the toxicity induced by MP, and the antioxidant defense mechanism is induced in bivalves, including *A. irradians*, because of the oxidative stress generated by exposure and accumulation of MP, such as in our study. In addition, increased oxidative stress may result in histological damage.

In summary, we found that scallops began to accumulate microplastics in the digestive diverticula within a day of becoming exposed and implemented oxidative stress responses which increased with concentration and exposure duration. After microplastic exposure for

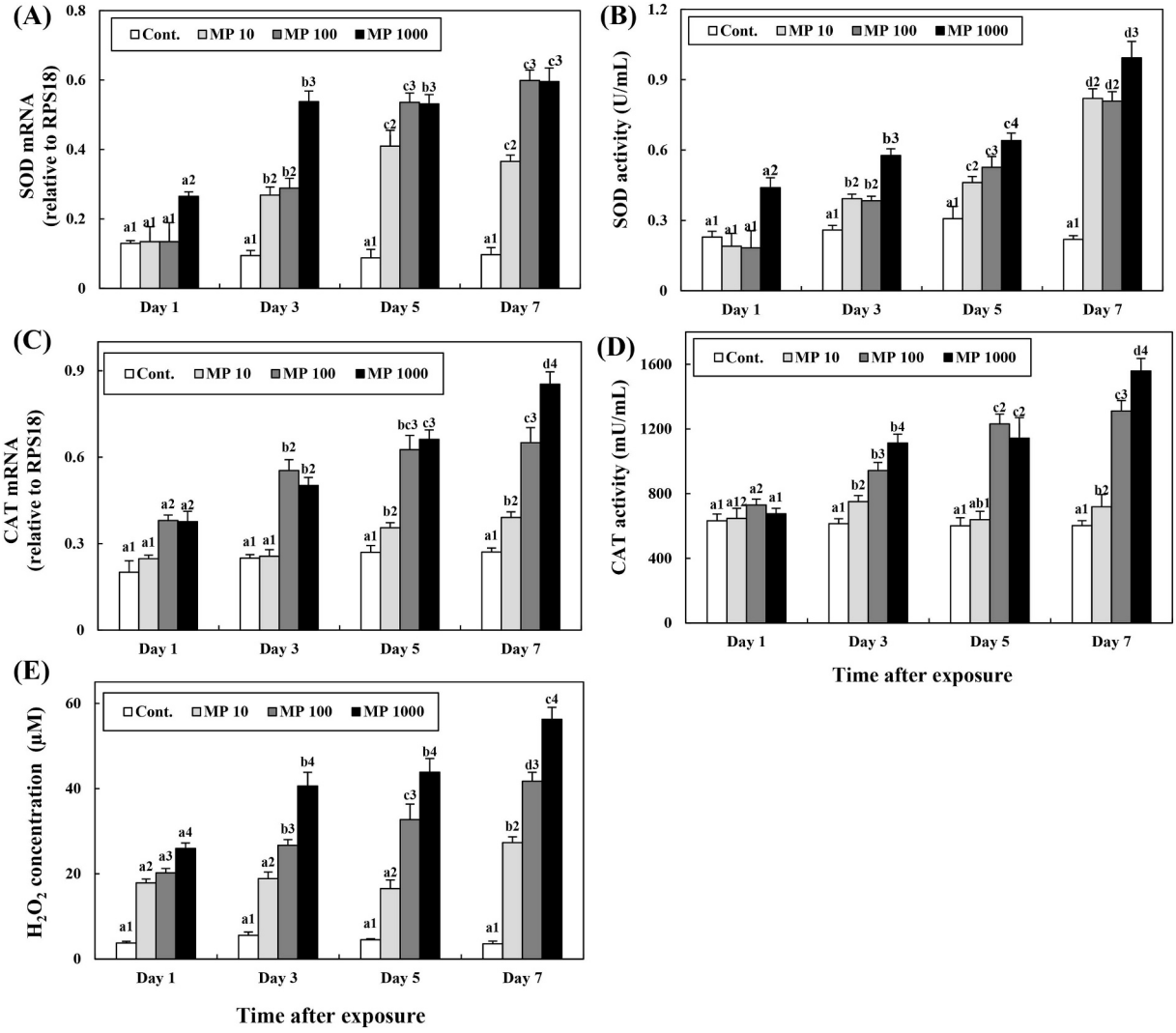

Fig. 2. (A) Changes in the expression of superoxide dismutase (SOD) mRNA in the digestive diverticula, (B) SOD activity in the hemolymph, (C) catalase (CAT) mRNA in the digestive diverticula, (D) CAT activity in the hemolymph, and (E) concentration of H_2O_2 in the hemolymph of *Argopecten irradians* exposed to microplastic beads for 7 days. Different letters indicate significant differences among scallops exposed to the same microplastic concentration for different durations ($P < 0.05$). Different numbers indicate significant differences among scallops exposed to different microplastic concentrations for the same duration ($P < 0.05$). Values indicate means ± SD ($n = 10$).

more than a day, *A. irradians* showed expression of antioxidant enzymes. This confirms a physiological response of *A. irradians* to 1 μm microplastics, suggesting that exposure to high concentrations and submicro-scale microplastics could have potential adverse effects on shellfish. Our results provide basic data on the effects of microplastics on bivalves. In the future, long-term monitoring will be required to draw clear conclusions about various bivalve species' endocrine responses to microplastic exposure.

Declaration of competing interest

The authors declare that they have no conflict of interests.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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