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Microplastic Beads Incorporated into a Single Cell: Analyses Using the Green Paramecium, *Paramecium bursaria*

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Abstract: The unicellular protist *Paramecium bursaria* harbors hundreds of symbiotic algae resembling *Chlorella* species in the cell. It is thought that the host *P. bursaria* uses some of photosynthetic products from these algae when sunlight is available. When photosynthesis cannot be performed, *P. bursaria* preys on bacteria, molds, algae, etc. in the surroundings for an energy source. Interestingly, some of the algae were observed to move from the food vacuole to the cytoplasm, becoming symbionts, within several days after incorporation into the cell. Since *P. bursaria* is benthic, it should be possible for various kinds of precipitated tiny particles to be taken up into the cell body during predation. In this study, microplastic (MP) beads with a diameter of 1 μm, which is about the same size as the algae, were mixed in the suspension medium of *P. bursaria*. It was observed that uptake of the beads into the cell body of *P. bursaria* started within 5 min after mixing, and the beads were observed inside *P. bursaria* even several days after the addition to the *P. bursaria* culture suspension. It is highly probable that the MP beads observed in the cell body somehow escaped from the food vacuole and moved into the cytoplasm.
Keywords: protist, micro plastic bead, cell division, benthic, ciliate

Introduction

In recent years, an environmental problem has often been reported where "tiny particles" such as plastic beads flow into the ocean and are ingested by living organisms. Plastic particles are classified as microplastics (MP) or nanoplastics (NP) according to their size. They constitute the "MP problem" and the "NP problem", respectively.

In the sea, MP have been found in the calanoid copepod (*Neocalanus cristatus*) and the euphausiid (*Euphausia pacifica*) collected in the northeast Pacific Ocean[1)]. In the case of mussels (*Mytilus edulis*), cultured in a suspension of MP beads for 3 days, MP beads could be detected in the hemolymph[2)]. Furthermore, it has been confirmed that when European green crabs (*Carcinus maenas*) prey on mussels that have ingested MP beads, the beads move from the intestinal tract of the crab into the ovary and gill[3)].

MP problems also exist in a freshwater environment. For example, in China, red belly tilapia and carp have been found to have MP beads in the gastrointestinal tract[4)]. In addition, MP beads have been found in major European rivers such as the Rhine and Danube, and it is conceivable that, MP beads may be found in freshwater fish from the Rhine and Danube in the future[5)].

These observations indicate the transfer of MP beads to the digestive tract of multicellular organisms such as fish and shellfish. Analysis of the process and subsequent effects of MP uptake will reveal extracellular effects of the incorporated MP beads. On the other hand, there have been few reports on the intracellular uptake of MP beads.

In this study, *Paramecium bursaria*, a benthic unicellular organism that feeds on submerged bacteria and algae at the bottom of freshwater sources, was

used to investigate whether *P. bursaria* incorporates MP beads that sink into the water. The localization of MP beads was investigated using a confocal laser microscope in *P. bursaria* inclividuals several days after exposure to MP beads.

Materials and Methods

P. bursaria

A cloned strain of *P. bursaria* collected from a pond on the Shonan Hiratsuka campus of Kanagawa University has already been established[6]. More specifically, a single *P. bursaria* was isolated from a pond water culture, washed, and grown in a lettuce culture medium prepared as follows to establish a cloned *P. bursaria* strain. Dried and sterilized lettuce leaves were extracted with distilled water (100 ℃ , 5 min). The extract (a lettuce culture medium) was autoclaved and stored until use.

MP beads

MP beads (Fluoresbrite®, 1.0 μm in diameter, yellow green polystyrene microspheres, Polysciences) were placed in an Eppendorf tube, and 70% (v/v) EtOH was added to 1 ml. The suspension was centrifuged in a small centrifuge for 5 min (2,000 g), and the supernatant was discarded. One ml of lettuce culture medium was added and the suspension was centrifuged again for 12 min. The supernatant was discarded. This 12 min centrifugation was performed three times in total. Lettuce culture medium was added to the resultant pellet of MP beads to obtain a lettuce culture medium containing MP beads.

P. bursaria culture in the presence of MP beads

Lettuce culture medium containing MP beads was prepared and *P. bursaria* was added. Three hundred μl aliquots of these suspensions (1.2×10^5 particles/cell) were separately put into individual wells of a 48-well plate (IWAKI microplate), and culture was performed in a BioTron incubator at 23℃ with alternating 12 hrs light / 12 hrs dark performed.

Tomography of *P. bursaria* using confocal laser scanning microscope (LSM)

P. bursaria were cultured in the incubator for several days in the presence of MP beads (3.1×10^7 particles in 300 μl) at a density of 860 cells/ml. In this case, the number of MP beads per single *P. bursaria* was 1.2×10^5. After the culture, the *P. bursaria* suspension was placed in an Eppendorf tube, and $NiCl_2$ was added at a final concentration of 0.1 mM as an anesthetic. The culture suspension, 16.2 μl, was removed from the Eppendorf tube and mounted on a slide glass. A cover glass (18 mm × 18 mm) was placed on the slide and sealed, and then tomography was performed on *P. bursaria* at every 1 μm using a confocal laser scanning microscope (LSM, Zeiss) to determine if MP beads had been incorporated into the cell body.

Results and Discussion

A previous report revealed phagocytic uptake of fluorescent nanospheres (0.6 μm) by marine heterotrophic flagellates and ciliates[7]. However, in these experiments using conventional microscopy, it seemed difficult to distinguish whether particles were inside the cell or just on cell surface.

P. bursaria cultured with MP beads for several days was imaged using LSM tomography. The tomography was performed from the dorsal (upper) side (panel A) to the ventral (bottom) side (panel O) of *P. bursaria* at a thickness of 1 μm to obtain 15 images (Fig.1). The green bead observed in Fig. 1 (indicated by the arrow) was confirmed only in the images shown in panels F and G. This indicates that MP beads seen were not present on the cell surface or in the culture suspension but were incorporated into the *P. bursaria* cell body. Within 5 min after mixing, 30% or more of *P. bursaria* were found to have incorporated MP beads (data not shown). This result indicates that *P. bursaria* is capable of incorporating the MP beads into the cell body immediately after contact with the beads.

It has already been shown that *P. bursaria* take up green algae having a diameter of about 1 to 8 μm [8] into the food vacuole, and that, within a few days, the incorporated green algae start symbiosis in *P. bursaria* after moving from the food vacuole to the cytoplasm, thus becoming symbiotic algae[9]. The diameter of MP beads used in this study was 1 μm, an appropriate size for *P. bursaria* to incorporate into the cell. In this study, MP beads were observed inside *P. bursaria* even several days after the addition to the *P. bursaria* culture suspension. Therefore, it is highly probable that the MP beads observed in the

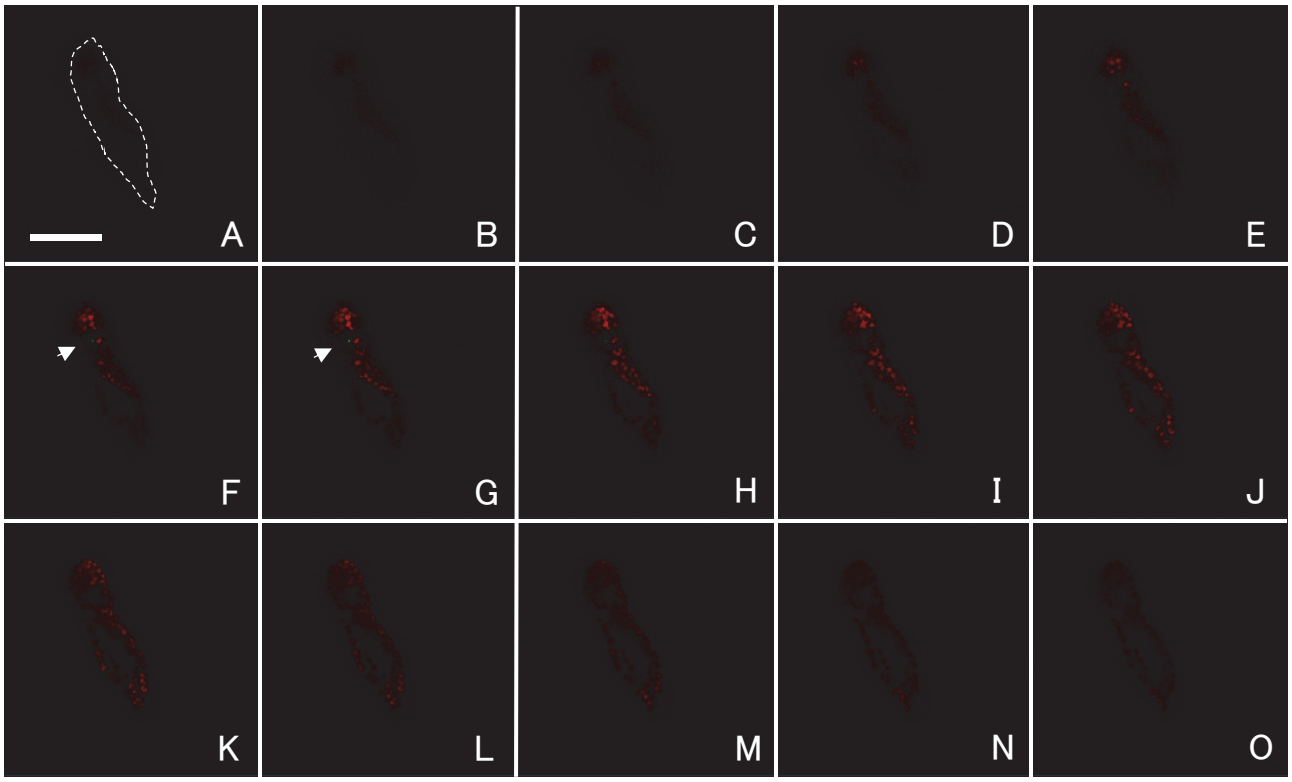

Fig. 1. Serial fluorescence images of *P. bursaria* with MP beads (1.0 μm in diameter) taken every 1 μm obtained by LSM (panels A-O). An MP bead (green dot) is observed in the panels F and G (indicated by arrows), showing that the bead was incorporated into the paramecium. The area surrounded by the white dotted line in panel A shows the original cell shape of *P.bursaria*. Scale bar, 50 um.

cell body somehow escaped from the food vacuole and moved into the cytoplasm. However, it remains unknown whether MP beads were first incorporated into the food vacuole or whether they were incorporated into *P. bursaria* by another mechanism not involving the food vacuole.

In the natural environment, chemicals are adsorbed on MP beads, and there are reports of the accumulation of such chemical substances in the body of multicellular organisms that eat MP beads by mistake. In the future, in addition to the effects of the uptake itself, it is necessary to study the effects of the chemical substances adsorbed onto MP beads when MP beads are taken up by unicellular organisms.

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