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Laboratory evaluation of floating marine plastic debris as a potential vector for transportation of the harmful benthic dinoflagellate *Fukuyoa koreansis*

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Abstract
Marine plastic debris (MPD) can significantly impact marine ecosystems because it can function as a dispersal vector for organisms, including toxic and alien species. We performed laboratory experiments to assess the possible function of MPD as a dispersal vector for the harmful epiphytic dinoflagellate *Fukuyoa koreansis*. Specifically, we monitored growth of these cells under 6 conditions: no MPD (control; with or without agitation), with polyethylene (PE) film (sheet-like MPD; with or without agitation), and with polypropylene (PP) rope (cylindrical MPD; with or without agitation). Growth without agitation was not significantly different between experimental groups ($p > 0.05$, $\chi^2 = 0.228$, Kruskal-Wallis test), indicating that the presence of MPDs had no significant effect on growth of *F. koreansis* under non-agitating conditions. After 15 days, growth without MPD was 25-fold greater without agitation than with agitation (150 ± 42 cells mL^{-1} *vs.* 6 ± 1 cells mL^{-1}). When grown with both floating MPDs and agitation, 78 ± 1 % of the cells were attached to the MPD, which was 4-times greater than when they were grown without agitation. These results suggest that MPD or another attachment substrate is essential for the growth of *F. koreansis* in an unstable water mass, and that MPD may provide a habitat or shelter for *F. koreansis* and thereby function as a dispersal vector for this harmful epiphytic dinoflagellate.

Keywords Marine plastic debris · Ciguatoxins · Epiphytic dinoflagellate · *Fukuyoa koreansis* attachment behavior · Potential vector

Introduction

Marine epiphytic dinoflagellates can attach to various macroalgal substrates that are widely distributed in tropical and subtropical coastal waters. *Fukuyoa* (an epiphytic dinoflagellate recently distinguished from a similar genus *Gambierdiscus*) produces ciguatoxins (CTXs) and maitotoxins (MTXs) as secondary metabolites, and these toxins are the primary cause of ciguatera fish poisoning (CFP) (Kohli et al. 2015; Litaker et al. 2017; Tester et al. 2018). Fish can be contaminated with high levels of CTXs through bioaccumulation, and consumption of these fish can be harmful to human health (Hurbungs et al. 2002; Chinain et al. 2010; Fraga et al. 2011). CFP caused by CTXs is responsible for toxic effects in 25,000 to 500,000 people annually around the world (Ragelis 1984; Fleming 1998). Thus, CFP caused by *Gambierdiscus* and *Fukuyoa* has become an important global issue because it poses a serious threat to public health and the sustainability of aquatic ecosystems (Bagnis et al. 1980; Chinain et al. 2010; Lee et al. 2014). Recent studies suggested that the habitats of harmful epiphytic dinoflagellates, including *Gambierdiscus*, had significant spatial heterogeneity and complexity (Yong et al. 2018; Mustapa et al. 2019). The recent increase in water temperature has led to reports of *Gambierdiscus* and *Fukuyoa* near Jeju Island (Korea), and this could be a threat to these marine ecosystems (Kim et al. 2011; Baek 2012a; Jeong et al. 2012). However, it is unclear how these dinoflagellates were transported from tropical and subtropical waters into the temperate waters near Jeju Island.

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Potentially harmful benthic dinoflagellates can attach to different marine biological substrates, such as drifting mangrove debris and macroalgae (Besada et al. 1982; Bomber et al. 1989; Faust and Gulledge 1996). In addition, epiphytic dinoflagellates such as *Ostreopsis* and *Coolia* can attach to the surface of marine plastic debris (MPD) (Masó et al. 2003; Larsson et al. 2018; Tibiriçá et al. 2019; Casabianca et al. 2019). MPD is now a major concern because it accounts for 80% of all marine debris worldwide (Gold et al. 2013; UNEP 2014). About 8 million tons of plastic materials are introduced into the marine environment each year. In 2014, researchers estimated that the total amount of MPD was 50 million tons, and that it will increase to 150 million tons by 2025 (Eriksen et al. 2014). It is now established that MPD affect neustonic, pelagic, and benthic habitats (Law et al. 2010; Lobelle and Cunliffe 2011; Zettler et al. 2013). Most MPD is buoyant and easily dispersed by wind and ocean currents (Zarfl and Matthies 2010). Given the propensity of epiphytic organisms such as *Gambierdiscus* and *Fukuyoa* species to attached to substrates, we propose that these organisms can attach to and drift with floating MPD. Although it is important to understand the role of MPD as a potential vector for the dispersion of harmful epiphytic organisms, no study has yet examined the attachment of dinoflagellates that are responsible for CFP, including *Gambierdiscus* and *Fukuyoa*, to MPD.

We hypothesized that the epiphytic dinoflagellate *Fukuyoa koreansis*, described as a new toxic species by Li et al. (2021), could have been introduced to temperate waters due to its attachment to MPD. To test this hypothesis, we performed laboratory experiments to determine if *F. koreansis* could attach to different types of MPD — polyethylene (PE) and polypropylene (PP) — and used agitation to simulate ocean turbulence.

Materials and methods

Isolation and cultivation of *F. koreansis*

Epiphytic dinoflagellates attached to macroalgae were harvested using the method described by Baek (2012a). Individual cells of *Fukuyoa koreansis* were isolated by Dr. Baek from natural assemblages of attached macroalgae at Seopseom (near Jeju Island) in June 2012, when the water temperature was 22°C and the salinity was 32.5 (Fig. 1A and B). The sample was transported rapidly to the laboratory for isolation. Cells were cultivated in a 250-mL culture flask (SPL, Korea) with F/2 medium (Guillard 1975) containing natural seawater (salinity of 32) that was passed through a 0.2 µm mixed cellulose ester membrane filter (Advantec, Japan) and then autoclaved. The culture was incubated at 24°C, with a 12 h light:12 h dark cycle and a photon flux density (PFD) of 60 µmol photons m^{-2} s^{-1} (cool white fluorescent lamp).

Growth and attachment experiment of *F. koreansis* on MPD

The PE and PP are buoyant polymers (low density) that make up plastic bags and ropes and are the dominant plastic litter in the aquatic environment (Ostle et al. 2019; Schwarz et al. 2019). The attachment of *F. koreansis* to an MPD sheet that had a smooth surface (PE film) and to an MPD cylinder that had a micro-filamentous surface (PP rope) was evaluated while cells were incubated with and without agitation (Fig. 1C). The agitation (12 rpm) was achieved using a Roller-Mixer (205RMC; Hwashin Instrument Co., Ltd., Korea) that provides mixing by rocking and rotating. For preparation of MPD, PE film with a thickness of 0.05 mm was cut into 2 × 4 cm rectangles (total surface area: 16 cm^2), and PP rope with a diameter of 0.4 cm was cut to a length of 4 cm (total surface area: about 6.7 cm^2) (Fig. 1D and E). The initial cell density was established at 30 cells mL^{-1} by dilution of a stock culture (600 cells mL^{-1}) in 22 mL of autoclaved filtered seawater with f/50 medium (25-fold dilution of f/2 medium) in a 25 mL glass vial. There were 6 different treatment groups, with 3 replicates per group: no agitation + no MPD; no agitation + PE film; no agitation + PP rope; agitation + no MPD; agitation + PE film; agitation + PP rope. All cells were incubated in experimental vials at 25°C, a salinity of 32, and a PFD of 60 µmol photons m^{-2} s^{-1} (cool white fluorescent light) with a 12 h light:12 h dark cycle (Photometer HD2101.1, Delta Ohm SrL, Caselle, Italy).

To monitor changes in cell density suspended in the medium, a 1 mL sample was collected every 2 days. At that time, it was considered that the cells attached to the bottom of the vial did not detach only by gentle mixing for homogenization of cells in the medium. After sample collection, an equal volume of autoclaved fresh f/50 medium was added into the vial to maintain the initial volume. The experiments were terminated on the 15th day, and cell colonies were visibly attached to the MPD at that time. At the end of the experiments, the total abundance of cells in the medium and on the MPD was determined. The PE film was removed using tweezers and placed in a Sedgewick-Rafter counting chamber, and cells were counted immediately without fixation using light microscopy (100×; Axio Scope A1; Carl Zeiss, Germany, Fig. 1F). The PP rope was transferred to a 15 mL conical tube (SPL Life Science, Korea) that contained autoclaved filtered seawater. It was then fixed with 3% Lugol's solution, and all attached cells were removed by vortexing. After removal of the PP rope, cells that were attached were also examined using stereomicroscopy (40 to 80×). The cell density in fixed samples was determined using light microscopy (100×). After MPD removal, total cell abundance in the medium on the 15th day was determined by fixing with 3% Lugol's solution and counting using light microscopy

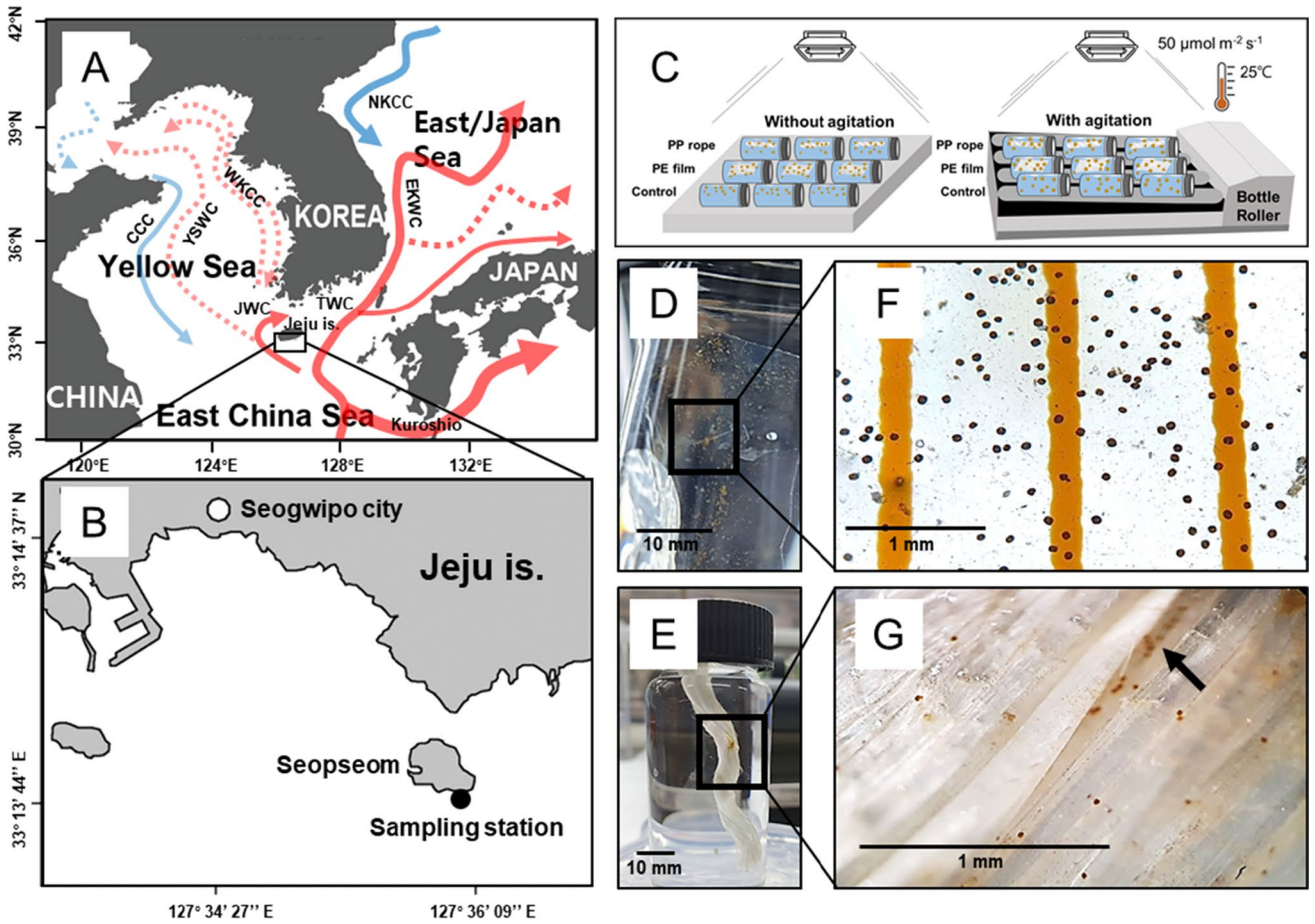

Fig. 1 Study area and experimental design. **A** Surface ocean currents in the Yellow Sea, East China Sea, Korea strait, and East/Japan Sea, showing the Kuroshio Current, Tsushima Warm Current (TWC), Jeju Warm Current (JWC), Chinese Coastal Current (CCC), Yellow Sea Warm Current (YSWC), West Korean Coastal Current (WKCC), East Korean Warm Current (EKWC), and North Korean Cold Current (NKCC). **B** Location of the sampling station where *F. koreansis* was isolated from (south of Jeju Island). **C** Design of the experiments, in which there were 6 experimental groups (no agitation + no MPD; no agitation + PE film; no agitation + PP rope; agitation + no MPD; agitation + PE film; agitation + PP rope) with 3 replicates per group, and cells were incubated at 25°C, and photon flux density of 60 µmol m^{-2} s^{-1} with a 12 h light:12 h dark cycle. **D** Attachment of cells to PE film (scale bar: 10 mm). **E** Attachment of cells to PP rope (scale bar: 10 mm). **F** Microscope image of cells attached to PE film (scale bar: 1 mm) using light microscopy (100×) **G** Microscope image of cells attached to PP rope (scale bar: 1 mm) using stereomicroscopy (80×)

(100×). At that time, the cells unattached to the MPD were considered to be suspended in the medium. To compare the growth of *F. koreansis* cells with and without agitation, non-parametric Kruskal–Wallis test was applied.

Results

Changes in cell densities of *F. koreansis* in medium

Growth under control conditions (no MPD and no agitation) led to a gradual increase of cell density, with a maximum of 150 ± 42 cells mL^{-1} on the 15th day (Fig. 2A). The growth in control initially showed the slowest but reached the highest cell densities by 14th day approximately 2-3 times the MPD treatments. Growth with PE film and no agitation steadily increased until the 12th day, reaching a maximum overall abundance of about 41 ± 9 cells mL^{-1} on the 12th day, and had similar densities on the 15th day (40 ± 10 cells mL^{-1}). Growth with PP rope and no agitation led to a cell density of 12 ± 2 cells mL^{-1} on the 8th day, followed by a rapid increase to 42 ± 7 cells mL^{-1} on the 10th day and 78 ± 42 cells mL^{-1} on the 15th day (Fig. 2A). Growth with agitation was significantly different in three groups (Control and PP rope > PE treatment; $p < 0.05$, $\chi^2 = 14.563$, Kruskal-Wallis test). The average cell density with agitation was 11 ± 3 cells mL^{-1} when there was no MPD, and appeared to not grow (Fig. 2B). The abundance of *F. koreansis* cells grown with agitation and with different types of MPD gradually increased until the 12th day, but remained low at less than 20 cells mL^{-1} by the 15th day.

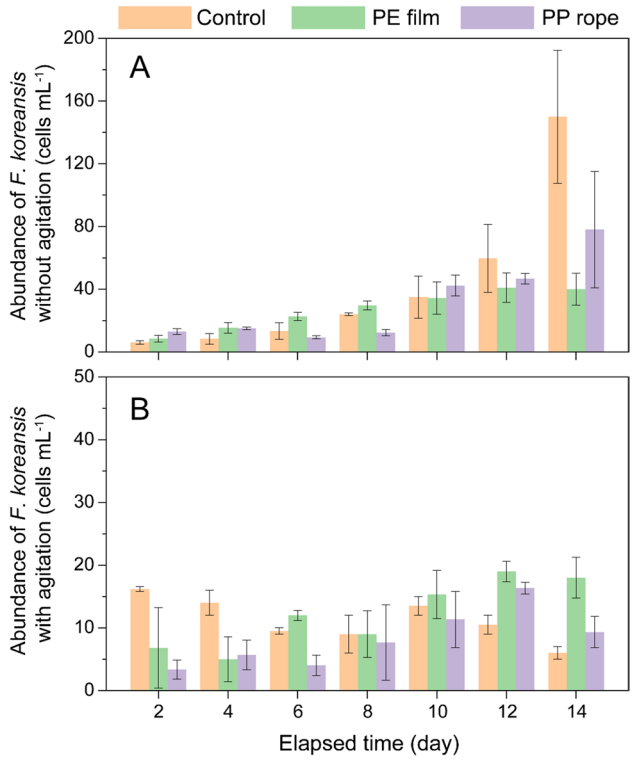

Fig. 2 Growth of *F. koreansis* cells without agitation (**A**) and with agitation (**B**) without MPD (orange), with PE film (green), and with PP rope (purple). Y-axis scale is different from (**A**) and (**B**), the y-axis scale of (**A**) is 4 times larger than (**B**). Error bars represent standard deviation (n=3)

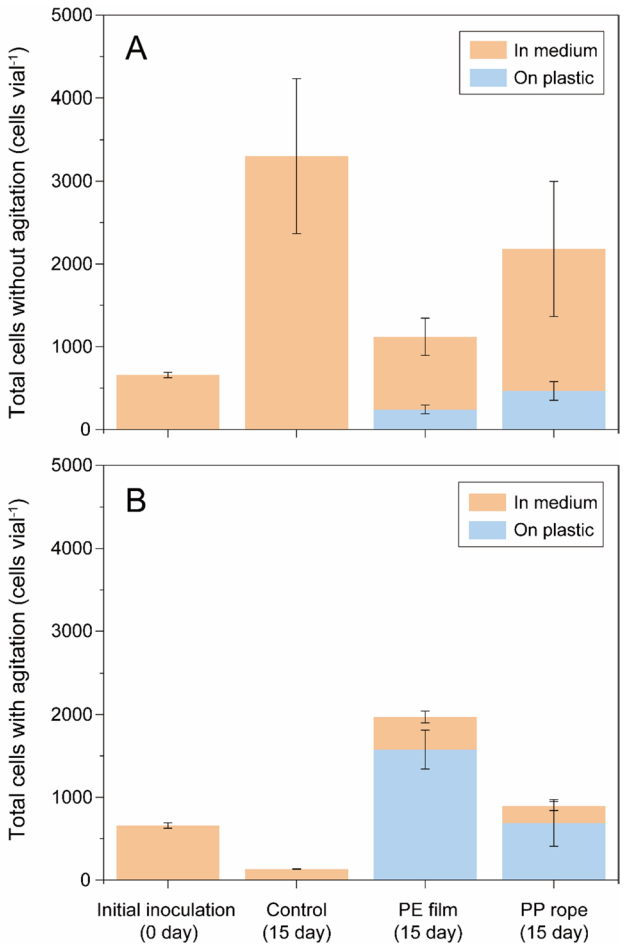

Fig. 3 Total abundance of *F. koreansis* cells grown without agitation (**A**) and with agitation (**B**). Cell abundance was determined in the growth medium (orange), on the PP film (blue), and on the PE rope (blue) at the beginning of the experiment (day-0) and the end of the experiment (day-15). Error bars represent standard deviation (n=3)

Changes in total abundance of *F. koreansis* in medium and MPDs

For incubation without agitation on the 15th day (Fig. 3A), growth without MPD, with PE film and PP rope led to a total abundance of 3.3 ± 0.9 × 10^3 cells, 1.1 ± 0.1 × 10^3 cells (78% in medium, 22% attached to MPD), and 2.2 ± 0.5 × 10^3 cells (79% in medium, 21% attached to MPD), respectively. The total abundance was 132 ± 4 cells when there was agitation but no MPD (Fig. 3B), about 25-times lower than growth without agitation and without MPD. In the presence of PE film with agitation, the total abundance was 2.0 ± 0.2 × 10^3 cells, higher than when cells were grown with PE film and without agitation. In addition, approximately 80% of the cells were attached to the PE film when they were grown with agitation (1576 ± 236 cells), almost 4-times higher than when cells were grown without agitation and with PE film. In the presence of PP rope, the total abundance was 0.9 ± 0.2 × 10^3 cells when there was agitation, lower than when cells were grown without agitation and with PP rope. However, a much higher percentage of cells were attached to the PP rope when there was agitation (77%, 690 ± 281 cells).

Discussion

Water temperature has a crucial role in the population dynamics and distribution of epiphytic and planktonic dinoflagellates (Baek et al. 2008; Lim et al. 2021; Li et al. 2021). To exclude the influence of temperature, all experiments with *F. koreansis* were conducted at 25°C, the optimal growth temperature for this species (Li et al. 2021). We assessed the attachment of *F. koreansis* cells to the different types of MPD using microscopy (Fig. 1F and G).

Growth with agitation led to lower cell densities when there was no added MPD substrate (Fig. 2B). The highest cell density in the control with agitation was 16 ± 7 cells mL^{-1}, which was 10-times lower than without MPD and no agitation. On the other hand, for incubation with agitation, the majority of cells (about 80%) were attached to the MPDs, about 7-times higher abundance than when cells were grown without agitation. According to Nakahara et al. (1996), cells

of a similar dinoflagellate (*Gambierdiscus*) that are swimming near macroalgae tend to quickly attach to them when there is a sudden disturbance or strong water movement. Moreover, another epiphytic dinoflagellate, *Ostreopsis*, exhibited higher abundance under conditions of moderate turbulence than strong turbulence (Vila et al. 2001; Totti et al. 2010; Selina et al. 2014). According to our results, the difference in total abundance was clear depending on whether MPD was present, and when *F. koreansis* cells were grown with agitation (total abundance in control < in PE and PP treatments), indicating that the floating MPD may serve as a stable environment to *F. koreansis* for growth under disturbance conditions by agitation. Therefore, these findings imply that unstable environment (continuous water mixing at 12 rpm) is not favorable toward the growth of *F. koreansis*, and an attachment substrate is required to effectively overcome unstable conditions.

Conversely, growth without agitation was not significantly different between the three groups ($p > 0.05$, $\chi^2 = 0.228$, Kruskal-Wallis test), indicating that the presence of MPDs had no significant effect on growth of *F. koreansis* under non-agitating conditions. In addition, for incubation without agitation, the number of cells unattached to MPDs were greater than the number of cells attached to MPDs (Fig. 3). During growth without agitation, the majority of cells (80%) were in the medium or the bottom of the vial. According to Nakahara et al. (1996), the epiphytic dinoflagellate *G. toxicus* usually swims around macroalgae after disturbance becomes weak, indicating that substrate attachment is not essential in a stable environment. Unlike unstable incubation by agitation, the cells of *F. koreansis* are relatively free from attachment without agitation. In addition, less energy is required to attach to the bottom than to floating MPD when there is no agitation. These may be the reasons why the rate of *F. koreansis* attached to MPD without agitation is lower than with agitation.

When cells were grown with agitation, similar numbers of cells were attached to PE film and PP rope (98 ± 15 cells cm^{-2} *vs.* 103 ± 42 cells cm^{-2}; $p > 0.05$, Mann-Whitney test) (Supplementary Table 1). Alternatively, when cells were grown without agitation, there was a significant difference in the number attached to PE film and PP rope, with the number of cells on PP rope 5.6 times higher (15 ± 3 cells cm^{-2} *vs.* 85 ± 20 cells cm^{-2}; $p < 0.05$, Mann-Whitney test). This could be attributed to the considerable difference between the surface structure of the PE film (which has a smooth surface) and the PP rope (which has a coarse surface). Thus, without agitation, it may be easier for cells to attach to the coarse surface of the PP rope than the smooth surface of the PE film. This also indicates that in the absence of agitation, cells appeared to have some intrinsic tendency for attachment to substrates. Numerous field studies showed that the highest abundance of the similar epiphytic dinoflagellate species *Gambierdiscus* is on micro-filamentous algae (Yasumoto et al. 1979; Bomber et al. 1989; Nakahara et al. 1996; Cruz-Rivera and Villareal 2006; Mustapa et al. 2019). This microfilament-structured surface provides shade, providing more low light growth condition which is more favorable (Bomber et al. 1988a; Morton et al. 1992). In addition, the surface structure of MPDs, similar to macroalgae, can provide shelter and a reduced micro-flow rate to epiphytic dinoflagellates so they are not disturbed (Gregg and Rose 1982; Tindall and Morton 1998). Therefore, when *F. koreansis* cells are grown without agitation, they are more likely to attach to a course surface than a smooth surface.

The rise in sea water temperature due to global warming has expanded the ranges of many potentially harmful epiphytic dinoflagellates, including *Gambierdiscus*, *Fukuyoa*, *Coolia*, *Prorocentrum*, and *Ostreopsis*, which are primarily endemic to tropical and subtropical regions (Penna et al. 2005; Aligizaki and Nikolaidis 2006; Parsons et al. 2012). The survival and spread of these toxic benthic dinoflagellates, including *G. toxicus*, *C. monotis*, *P. lima*, *O. heptagona* and *O. siamensis* by natural vector drifting macrophytes has been reported (Bomber et al. 1988b; Larsson et al. 2018). In addition, the attached cells of *Coolia* sp., *P. lima* and *O. ovata* have been detected on floating MPDs (Masó et al. 2016; Casabianca et al. 2019; Tibiriçá et al. 2019). These findings indicating that MPD can act as an artificial vector, in addition to other natural vectors, which can then accelerate the spread of these various toxic epiphytic dinoflagellates. In particular, Jeju Island in Korea is in a temperate region, but its coastal waters are affected by the Tsushima Warm Current (Fig. 1A), which introduces tropical fish, invertebrates, and epiphytic dinoflagellates into this region (Baek 2012a; b; Son et al. 2020). In addition, foreign-origin MPD are frequently found in the southwestern coast of Korea and Jeju Island (Jang et al. 2012; Seo and Park 2020). MPD contain various microbial heterotrophs, autotrophs, predators, and symbionts, and may therefore constitute a "plastisphere" (Zettler et al. 2013). Our experiments suggest that MPD can transport epiphytic dinoflagellates such as *F. koreansis* as part of this plastisphere. Because the temperate waters off Jeju Island are favorable for the growth of epiphytic dinoflagellates (Lee and Park 2020; Li et al. 2021), it is possible that the introduction of harmful epiphytic dinoflagellates will significantly disrupt this ecosystem. To prevent the damage caused by these intrusions, it is necessary to monitor the introduction of subtropical and tropical species using additional on-site surveys, investigate attachment of transported epiphytes, and examine the plastispheres that occur on MPD transported into the area.

Our laboratory experiments demonstrated that an attachment substrate is essential for the growth of *F. koreansis* in an environment with an unstable water mass. Our results

indicate that the attachment of *F. koreansis* to drifting MPD may play a role in the dispersal of this species. Therefore, drifting MPD functions as a plastisphere and may pose a potential hazard because it can function as a vector of epiphytic organisms, including those responsible for CFP.

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Author contribution Young Kyun Lim: Conceptualization; Experiment; Formal analysis; Writing original draft; Visualization, MinJi Lee: Experiment; Visualization, Seong Jin Hong: Writing- review & editing; Validation, Seung Ho Baek: Conceptualization; Funding acquisition; Writing- review & editing; Validation

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary material

Supplementary Table 1. Number of *F. koreansis* cells attached on MPD (PE film and PP rope) without agitation and with agitation at end of the experiment on the 15th day.

Agitation	MPD	Surface area (cm^2)	Total cells attached on MPD (cells)	Cells attached on MPD per unit area (cells cm^{-2})
Without agitation	PE film	16	242 ± 52	15 ± 3
	PP rope	6.7	467 ± 113	85 ± 20
With agitation	PE film	16	1576 ± 236	98 ± 15
	PP rope	6.7	690 ± 281	103 ± 42