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Eukaryotic community succession on discarded face masks in the marine environment

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ABSTRACT

Wearing facemasks remains an essential strategy for combating the COVID-19 pandemic. However, used masks are becoming plastic wastes that are widespread in the oceans, which is raising concerns about the potential impacts of these novel plastic niches on marine organisms. To delve into this issue, we exposed surgical masks to coastal waters for 30 days. Valuable information was recorded weekly in regard to the succession of the eukaryotic community inhabiting the masks via high-throughput 18S rRNA gene sequencing. Generally, the community on masks was significantly distinct from that in the surrounding seawater. With 1150 different eukaryotic taxa identified, the diversity of the vigorous colonizers of masks peaked at the beginning and decreased over time. A hallmark of initial colonization was the aggregation of diatoms, which formed biofilms on masks, followed by dinoflagellates that acted as a turning point for subsequent development of calcified species and other predators. This study provides insight into the eukaryotic community dynamics on discarded masks in the marine environment and highlights that the potential mask-mediated harmful species clustering may threaten the marine ecosystem.

1. Introduction

Since the outbreak of COVID-19, face masks have become essential to fight against the pandemic. Around 129 billion face masks are used monthly, which is unavoidably exacerbating the risk of waste mismanagement, and a substantial number of discarded masks have already been found in urban and natural environments (Adyel, 2020; Prata et al., 2020; Ammendolia et al., 2021). Multiple pathways lead to the discarded masks ending up in nearshore regions, and they have become an emerging source of secondary microfibers and microplastic particles (Wang et al., 2021).

Spun-bond or melt-blown micro polypropylene (PP) fibers are the most common material used to manufacture the three layers of a typical surgical
face mask (Parashar and Hait, 2020; Xu and Ren, 2021). The fibrous structure gives masks a much higher surface area per unit mass than traditional plastic products such as films or bottles (Miličky et al., 2021). Nevertheless, PP has a density of 0.89–0.92 g cm$^{-3}$, which is lower than that of both freshwater and seawater with densities beyond 1.00 g cm$^{-3}$ (Andray, 2015). This enables masks to remain afloat at the ocean surface, serving as an ideal substrate for colonization by marine organisms in the euphotic layer.

Microbial communities can colonize plastics in seawater in what is referred to as the marine “plastisphere,” where they go through distinct stages of succession (Datta et al., 2016; Pollet et al., 2018). Recent publications have highlighted the non-ignorable role of microplastics as a hotspot for diverse pollutants (heavy metals, organic pollutants, etc.) within the plastisphere (Gao et al., 2019; Santana-Viera et al., 2021; Liu et al., 2022; Tang et al., 2022), which poses composite risks to nearby organisms and may potentially complicate the dynamics of biological colonization. Although the composition of the thriving colonizers of plastics often differs from that of natural surfaces or surrounding water at early stages, all these communities tend to converge over time (Enri-Cassola et al., 2019; Wright et al., 2020).

Eukaryotic communities in the plastisphere have been less explored. Marine diatoms are identified as one of the initial colonizers on plastic litter (Sweat and Johnson, 2013). They can easily stick to natural and anthropogenic surfaces, probably due to their high surface-to-volume ratios and abundant secretion of extracellular polymeric substances, which pave the way for biofilm formation. Afterwards, diatoms may enable the settlement of heterotrophic species and the larva of macrofouling organisms (Dobretsov et al., 2013; Kettner et al., 2019).

Notably, the abundance of eukaryotic colonizers could result in buoyancy loss and sinking for the floating plastics (Kaiser et al., 2017; Amaral-Zettler et al., 2021). Moreover, the attached biofilms make floating masks resemble gelatinous zooplankton such as jellyfish (Dharmaraj et al., 2021), which might increase the risk of mistaken ingestion of masks by marine animals and cause their mortality (Alexiadou et al., 2019; Neto et al., 2021; Fukuoka et al., 2022). Another novel aspect of eukaryotes is that zooplankton grazing on plastic debris have been estimated to decrease the global oxygen inventory and accelerate climate warming (Kvale et al., 2021). In general, the types of plastic and the exposure time are likely to play pivotal roles in the eukaryotic community in the plastisphere (Long et al., 2017; Dudek et al., 2020; Grassi et al., 2020). However, few studies have been devoted to uncover the dynamics of the eukaryotic community occupying discarded masks in a marine environment.

This study aimed to examine the colonization and succession of eukaryotes on discarded facemasks in natural seawater by SEM and high-throughput 18S rRNA gene sequencing. We expected dense colonization of biofouling eukaryotes can be observed on the mask fibers that have a much higher surface to volume ratio than bulk plastics. Our results revealed that diatoms were the initial colonizers of the mask surfaces. Afterwards, dinoflagellates made up a major proportion of the community. Nonselective heterotrophic/predator organisms were dominant throughout the exposure period, especially after the formation of a matured biofilm. This study provides important implications for the fate of discarded masks in the marine environment.

2. Materials and methods

2.1. Exposure of face masks to natural seawater

A batch of surgical masks was purchased from a local drug store. All of the masks were within the manufacturer’s designated shelf life. To observe the growth of eukaryotic biofouling organisms on the masks, a total of 100 masks were equally placed into 10 net bags, which were tied to a floating raft and kept submerged at a depth of 1 m in seawater in Dapeng Bay, China, on the eastern side of Hong Kong. Each week, 10 masks and surrounding seawater were randomly sampled and transported back to the laboratory.

2.2. Examination of colonized eukaryotic organisms on masks

Upon arrival at the laboratory, the masks were carefully cut into pieces with dimensions of 0.5 × 0.5 cm$^2$ and dehydrated through a graded ethanol series. The mask pieces were then critical-point dried, sputter-coated with a 3-nm layer of Pt, and visualized with a scanning electronic microscope (SEM, Tescan, MIRA3, Oxford, Czech Republic) at an accelerating voltage of 5 kV.

2.3. DNA extraction and amplicon sequencing

Genomic DNA was extracted from biofilm and seawater samples with a DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. Briefly, 0.5 g of mask sample was placed into a PowerBiofilm Bead Tube containing the extraction solution. The tubes were fixed on an orbital shaker to break cells and extract DNA. The supernatant in each tube was purified by a series of DNA-cleaning solutions, and final DNA was eluted in 50 μl. Qubit fluorometric quantification (Thermo Scientific) was used for DNA quantification, and each sample was diluted to equal concentrations of 10 ng μl$^{-1}$.

18S rRNA gene sequencing was carried out on the V4 region using the primer pair of Eu56SF and Eu981R (Stoeck et al., 2010). DNA was amplified by PCR with the following steps: denaturation at 95 °C for 3 min, 25 annealing cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and elongation at 72 °C for 4 min. The PCR amplicons were purified with AMPure XP beads, and the quantity of DNA was checked using a Qubit fluorometer. All PCR products were diluted to 0.2 ng μl$^{-1}$, and 6 μl of the products were sequenced on an Illumina MiSeq platform according to manufacturer’s instructions.

2.4. Determination of eukaryotic community structure

The sequencing data output from the Illumina MiSeq was processed using the Dada2 plug-in in R (Callahan et al., 2016). Both forward and reverse sequences were trimmed to a length of 220 base pairs to facilitate comparative analyses. Sequences were merged and aligned, and chimeras were filtered out. We used the database of Silva version 132 to match the taxonomy information (Yilmaz et al., 2014). R packages phyloseq, ggplot2, and vegan were used for statistical analyses and visualization.

2.5. Data analyses and statistical methods

Statistical comparison of differences between treatments was performed with one-way or two-way analysis of variance (ANOVA) using R Studio. The Shapiro–Wilks test was employed to test the normality of data as appropriate. A student’s t-test was used to determine which treatments differed significantly at p < 0.05. The Kruskal–Wallis test was performed to compare the significant difference in the alpha diversity (indices of Shannon’s diversity, Ace’s and Chao’s richness) between the treatments using the vegan package in R Studio.

3. Results and discussion

3.1. General view of the eukaryotic biofouling on masks in the marine environment

The growth of biofouling organisms on the mask fibers is shown in Fig. 1. Diatoms were the pioneering eukaryotic colonizers (Fig. 1B). After 14 days in seawater, a thin layer of biofilm and dinoflagellates appeared on mask fibers (Fig. 1C). The biofilms grew thicker on the fibers after 21 days, and an array of unidentified organisms was intertwined with the
films (Fig. 1D). After 30 days, the fibers were completely covered by thick biofilms (Fig. 1E).

Specifically, diatoms in pennate and centric groups were the most observable colonizers on mask surfaces (Fig. 1F to J), with the former accounting for a higher proportion. They either lay flat or erect on the fibers, indicating they may generate secretion for firm adhesion. We could still observe some diatoms after 14 and 21 days, but in much lower abundance. Calcified larvae of serpulid were common on mask surfaces after 14 days (Fig. 1K and L). Dinoflagellates were also found in high amounts after 14 days, and their resting cysts showed up after 21 days (Fig. 1M –N). In addition, we also spotted fragments of unidentifiable macroorganisms on the surface of the fibers (Fig. S1A, B), and these later-arriving predator species might ingest the first-arriving diatoms and generate feces comprising aggregates of frustule-like structures (Fig. S1C). The SEM observation may be limited by the scanning scale and the complexity of the matured biofilms. Thus, the accurate dominant species could only be revealed by high-throughput sequencing described below.

While masks have been identified to be viable substrates for the growth of microbials in marine environments (Crisaf et al., 2022; Ma et al., 2022), the eukaryotic community on masks has not been explored previously. Studies have shown that the typical fouling organisms on marine plastic wastes include algae, hydroids, barnacles, bryozoans, mussels, and ascidians (Fazey and Ryan, 2016; Gündoğdu et al., 2017; Crocetta et al., 2020). The biomass and community structure of colonizers are dependent on the type of plastics, especially at the early stage of colonization (Oberbeckmann and Labrenz, 2020; Póvoa et al., 2021).

Our SEM observations confirmed that various eukaryotic organisms can colonize the facemasks (Fig. 1). This is consistent with recent studies showing that eukaryotic colonizers play a key role in constructing plastic biofilms (Amaral-Zettler et al., 2021; Zhao et al., 2021). Eukaryotic colonizers may also cause changes in buoyancy and carbon biomass of the plastic substrates in coastal waters, which gives importance to investigating the composition and development of the eukaryotic community on masks.

3.2. Structure of the eukaryotic community on masks

The 18S rRNA gene sequencing revealed the succession of the eukaryotic community structure on the mask surface during 30 days of exposure in coastal seawater. A total of 1150 different eukaryotic taxa were identified, indicating the masks were inhabited by diverse species. While the eukaryotic community in surrounding seawater remained relatively stable, the community on masks underwent dramatic changes. Based on the sampling days, the Shannon, Ace, and Chao indices demonstrated that samples exhibited the highest and second highest alpha diversity in the eukaryotic community at 7 and 14 days, respectively. In comparison, the samples at 21 days and 30 days had the second lowest and lowest alpha diversity, respectively, which was even lower than that of the seawater samples (Table 1).

The number of operational taxonomic units (OTUs) detected also reflected the increasing differences among the samples over time (Fig. 2A). The surrounding seawater occupied 498 (75 unique) OTUs, while masks had 554 (108 unique) and 512 (183 unique) OTUs at 7 and

Table 1
The alpha diversity of the eukaryotic communities in terms of Shannon, Ace, and Chao diversity indices. Values plus or minus the standard error are presented.

|        | Shannon | Ace        | Chao        |
|--------|---------|------------|-------------|
| Seawater| 5.25 ± 0.03| 655.43 ± 44.15| 620.99 ± 42.35|
| 7 days  | 5.97 ± 0.04| 697.44 ± 33.67| 709.30 ± 44.97|
| 14 days | 5.92 ± 0.01| 685.93 ± 24.51| 696.64 ± 22.23|
| 21 days | 4.79 ± 0.02| 657.80 ± 53.91| 604.92 ± 42.44|
| 30 days | 3.86 ± 0.02| 440.50 ± 35.83| 454.38 ± 48.92|
14 days, respectively. Only 412 (73 unique) and 335 (60 unique) OTUs were identified on masks at 21 days and 30 days, respectively.

Principal coordinate analysis (PCoA) was used to compare the communities’ beta diversity. Dissimilarity was measured with a Bray–Curtis matrix, and the first two principal components explained 51.92 % of the total variance among the communities (Fig. 2B). Permutational multivariate analysis of variance (PERMANOVA) identified significant differences between these community dissimilarities (p = 0.001). All the communities on the masks were distinctly different from the seawater community in the top left quadrant (Fig. 2B). The community in the surrounding seawater showed high similarity through the 30-day sampling period (Fig. 2B and Fig. S2). The community at 7 days was in the bottom left quadrant, while the community at 14 days was in the bottom right. The communities at 21 and 30 days were similar to each other and were both clustered in the top right quadrant (Fig. 2B). This pattern emphasizes the time-dependent changes of the eukaryotic community on masks.

The masks showed higher community abundance and diversity at 7 and 14 days than the surrounding seawater. Similarly, previous studies have found more eukaryotic OTUs on the surface of PET bottles and PE/PS particles than in seawater (Oberbeckmann et al., 2016; Kettner et al., 2017).

Although Zettler et al. (2013) found that seawater samples had the most abundant OTUs, when normalized by the number of reads recovered, PP samples actually had the most abundant OTUs.

The abundance of taxa is related to the surface area of the substrate, so the highest community diversity is expected on the largest plastics (Zettler et al., 2013). Masks have huge surface areas, so this indicates that they might support the colonization of marine organisms with high abundance and diversity. The lower OTU abundance and diversity on 21-day and 30-day samples can be attributed to the community succession on masks, as several organisms gradually took over the mask surfaces (Datta et al., 2016; Wright et al., 2019).

3.3. The succession of eukaryotes on masks

Despite the contrasting community diversity, the 7-day samples of seawater and masks shared dominant species (relative abundances: 52.99 % and 33.11 %, respectively). These species included uncultured ciliate (Ciliophora), Gastrotrichus sp. (Ciliophora), and Pleurosigma sp. (Bacillariophyta, diatom) (Fig. 3). Then, a sharp change in dominant species was observed in 14-day samples, which were dominated by six Dinophyceae species (25.22 % abundance): Scrippsiella sp., Dissodinium pseudolunula, Biechleriopsis adriatica, Gymnodinium microreticulatum, and two unidentified dinoflagellates. Three other abundant species (19.28 % abundance) were also found: Dinovorax pyrififormis (Perkinsozoa), Halichona totoxus paradoxoxus (Gastrotricha), and Chaetoceros sp. (Bacillariophyta) (Fig. 3).

Fig. 2. Composition of eukaryotic community inhabiting masks after exposure in coastal seawater for 7, 14, 21, and 30 days. (A) Venn diagram showing the overlap in numbers of detected eukaryotic OTUs between the surrounding natural seawater and face masks sampled with increasing exposure time. The numbers represent the amounts of OTUs in this fraction. (B) Principal Coordinate Analysis (PCoA) plot showing Bray–Curtis distances to compare the communities’ beta diversity between eukaryotic microbial composition at different sampling times. Percentages displayed in the axis labels indicate the proportions of variation contributed by each axis. Different exposure periods are denoted with different colors and shapes (natural seawater was collected on day 0, 7, 14, 21, and 30 as the control group (green)).
Fig. 3. Bar chart showing variability of species number and abundance among samples. Species-level stacked bar graph showing relative abundance of the top-20 microbial taxa for each sample identified in the eukaryotic community of normal seawater and face masks collected at different exposure times. The most abundant OTUs are labeled as above (number 1 to 40).

Fig. 4. Co-occurrence and co-exclusion network of the eukaryotic communities inhabiting face masks after different exposure times at OTUs level. Dominant OTUs (top 100) were chosen to establish the network. Nodes representing OTUs are colored by their distinct abundant pattern (green: remarkably high abundance at 7 days; blue: remarkably high abundance at 14 days; orange: remarkably high abundance at 21 days; red: remarkably high abundance at 30 days; purple: no clear pattern of abundance throughout the exposure period). The size of a node is proportional to the number of connections. The red and grey edges in the network represent positive and negative correlations, respectively. The dotted ellipses represent four disconnected clusters within the network. Only strong (Spearman’s $\rho > 0.6$) and significant ($p$-value < 0.01) correlations are presented (details are in Table S1 in supporting information).
Notably, there were the numerous positive correlations among the OTUs with high abundance at 14 days (blue nodes in Fig. 4). Looking into the taxa related to these OTUs revealed strong co-occurrences and interactions of the dinoflagellates (OTUs 2, 46, 355, 530, 640, 661, 663, 688, 696, 936, 1460) (Fig. 4 and Table S1). This is consistent with observations in Fig. 3 and suggests that the colonization of dinoflagellates represent an important stage in the succession of the eukaryotic community.

Data from 18S rRNA gene sequence also provided evidence for biological phototrophy, symbiosis, heterotrophy, and predation (Fig. 5). The initially abundant diatoms on masks were gradually replaced as the community matured (Figs. 1, 3, and 5). This is consistent with previous studies that diatoms are ubiquitously distributed and one of the first colonizers on marine plastic surfaces (Amaral-Zettler et al., 2020). During their colonization, phototrophic diatoms excrete extracellular polymeric substances (EPSs), promoting the formation of biofilm and biogeochemical activity within it (Patil and Anil, 2005; Hanlon et al., 2006). A prior study has shown that the oxygen production rate is negative for the initial biofilm on marine plastic wastes, indicating oxygen consumption (Eich et al., 2015). Thus, the biofilms on plastics triggered by diatoms not only offer spaces, but also reserve biomass and energy such as DOC and nitrogen for the successional colonization (Zhou et al., 2017; Grassi et al., 2020).

Unsurprisingly, predatory species dominated the community throughout the exposure period (Fig. 5). As primary or secondary consumers, ciliates were detected in both surrounding seawater and the 7-day samples, which corresponds to these species’ roles as initial colonizers with less selectivity (Wang and Xu, 2015). Different from the bacterial community, in which the decomposer species have the potential to utilize and biodegrade plastic polymers, these heterotrophic species are more likely to ingest the fouling bacteria and algae or even mistakenly ingest the microplastic debris due to high similarity between plastic polymers and natural cellular surfaces (Procter et al., 2019; Kvale et al., 2021). However, caution is warranted since the high abundance of metazoans at 21 days and 30 days might be due to their multicellularity (Kettenr, 2019).

Tube-forming serpulid worms *H. elegans* appeared on the surfaces of masks after 21 days and became the most dominant species, tightly adhering to masks after 30 days (Fig. 1P and 3). This observation indicates that in a later stage of succession, *H. elegans* was well established and possibly reproduced on surfaces of masks, taking advantage of the formed biofilms. Consequently, their calcified tubular structures could contribute substantially to the negative buoyancy and sinking of masks (Thiel and Gutow, 2005; Nedved and Hadfield, 2008). Nevertheless, the calcified fouling organisms may release calcium into the microenvironment of plastics’ surfaces. Furthermore, they may influence non-specific interactions, such as neutralization of the electrical double layer between cells and substratum surface, as well as specific adhesive interactions that cannot be replaced by other cations (Geeseey et al., 2000; Grassi et al., 2020; Zhao et al., 2021).

4. Conclusions

Our results demonstrate the dynamics of eukaryotic mask colonizers for the first time, and their composition differs dramatically from that in the surrounding seawater. As demonstrated by SEM images, masks exposed to natural seawater can support the settlement and growth of diverse fouling organisms, in addition to possibly sheltering their reproduction and interaction (predation, parasitism, etc.). In brief, clusters of diatoms attached to masks and facilitated biofilm formation after one week, representing the initial stage of microbial colonization on masks. Then, dinoflagellates appeared (probably facilitated by diatoms) and outnumbered the diatoms, which acted as a critical turning point for subsequent development of calcified species and other predators. Notably, the potential aggregation of harmful algae on masks may pose ecological risks and is therefore deserve further exploration. Using a high-throughput sequencing approach, this study has revealed the general short-term succession pattern of the eukaryotic community inhabiting face masks, which are an emerging substrate in the marine environment.

CRediT authorship contribution statement

**Jie Ma**: Conceived and designed the research, performed the experiments and analyzed data, and wrote the manuscript. **Fengyuan Chen**: Performed the experiments, analyzed data, and wrote the manuscript. **Zhen Zhang**: Performed the experiments and analyzed data. **Yangping Li**: Performed the experiments. **Jingli Liu**: Analyzed data. **Tara Chun Chen**: Performed the experiment. **Ke Pan**: Conceived and designed the research, and wrote the manuscript.

Data availability

Data will be made available on request.

Declaration of 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.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (42076148, 41976140, 42006141), Science and Technology Innovation Commission of Shenzhen (JCYJ20180507182227257), Guangdong Basic and Applied Basic Research Foundation (2019A1515011630, 2022A1515010681), Guangxi Key R&D Program of China (GUlKE AB20297018), and the Innovation Team Project of Universities in Guangdong Province (No. 2020KCXTD023).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.158552.

![Fig. 5. Bar charts showing the succession of top-100 eukaryotic species after classification into prey/predator species. Both Bacillariophyceae and Dinophyceae were listed separately to emphasize their key roles in the succession.](image-url)
