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SPECIAL ISSUE-LETTER

Patterns of suspended and salp-ingested microplastic debris in the North Pacific investigated with epifluorescence microscopy

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Scientific Significance Statement

Marine debris is a worldwide ocean pollution problem and evidence suggests that the tiniest pieces of plastic in the ocean, microplastics, may be the most abundant forms of plastic pollution in the ocean; however, the smallest microplastics have yet to be accurately quantified due to methodological limitations. We successfully developed and tested a new method for collecting and counting the smallest microplastic pieces in seawater and ingested inside the guts of salps, a planktonic species at the base of food webs and key to transport of carbon and particles from the sea surface to the deep sea. We determined that the true abundance of these tiniest microplastics far outnumber previously reported counts, and that every salp we examined had ingested plastic.

Abstract

Microplastics (< 5 mm) have long been a concern in marine debris research, but quantifying the smallest microplastics (< 333 μm) has been hampered by appropriate collection methods, like net tows. We modified standard epifluorescence microscopy methods to develop a new technique to enumerate < 333 μm microplastics (mini-microplastics) from filtered surface seawater samples and salp stomach contents. This permitted us to distinguish mini-microplastics from phytoplankton and suspended particles. We found seawater mini-microplastic

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Author Contribution Statement: J.B. designed and conducted the research. A.F. provided expertise and input about microscopy methods and identification of microorganisms. L.M.S. performed salp identification and provided expertise on salp dissection and life history. J.B. conducted the data collection, processing, and analyses. During analyses, J.B. consulted with A.F. and L.M.S. on topics within their areas of expertise. J.B. wrote the majority of this paper, with some sections written and edited by A.F. and L.M.S.

Data Availability Statement: Data are available in the Datazoo repository at:

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- doi:10.6073/pasta/932232646478eb5be878965f0693dde4
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concentrations that were 5–7 orders of magnitude higher than published concentrations of > 333 μm microplastics. Mini-microplastics were the most abundant in nearshore waters and more evenly distributed from the California Current through the North Pacific Subtropical Gyre. Every salp examined had ingested mini-microplastics, regardless of species, life history stage, or oceanic region. Salps ingested significantly smaller plastic particles than were available in ambient surface seawater. The blastozooid stage of salps had higher ingestion rates than oozooids.

Marine debris is a worldwide ocean pollution problem, with plastics found in virtually all aquatic environments (Goldstein et al. 2013; GESAMP 2016). The majority of marine debris analyzed to date has been microplastic, plastic < 5 mm (Hidalgo-Ruz et al. 2012; Goldstein et al. 2013). However, findings suggest even smaller plastics (< 333 μm) are both under-sampled due to the inappropriate mesh size of common sampling nets (Van Sebille et al. 2015) and far more numerous because plastic particles physically degrade over time into progressively smaller pieces (Gilfillan et al. 2009).

Such small debris can be consumed by, and deleterious to, suspension-feeding marine organisms (Wright et al. 2013), including salps (Madin and Deibel 1998). Salps are pelagic tunicates that possess the highest per-individual filtration rates among marine zooplankton, ingesting particles from < 1.0 μm to 1.0 mm (Madin and Deibel 1998; Sutherland et al. 2010). They primarily feed in the upper water column, where microplastics are abundant (GESAMP 2016). Once plastics are ingested by zooplankton, they have the potential to bioaccumulate in the food web into larger organisms (Dawson et al. 2018), along with adsorbed persistent organic pollutants and harmful chemical additives (Ogata et al. 2009; Jang et al. 2016), with unknown physiological consequences.

Although many zooplankton species consume microplastic in a laboratory setting (Cole et al. 2013), the ecologically significant question lies in whether they are ingesting such particles in situ. Although the measured abundance of surface seawater microplastics is high (Law et al. 2010, 2014; Cózar et al. 2014; Eriksen et al. 2014), it is 1–3 orders of magnitude below model predictions of plastic inputs (Cózar et al. 2014; Jambeck et al. 2015). Smith et al. (2014) reported a considerable influx of both salp tunics and fecal pellets to ~ 4000 m depth following a bloom of *Salpa* spp. Thus, salps could be a key link explaining the discrepancy between modeled and measured abundances of buoyant plastics, because fast-sinking salp fecal pellets and carcasses may be a vector moving ingested surface microplastics to the deep sea.

We aimed to isolate, identify, and quantify microplastics 5–333 μm in size, a subgroup of microplastics which we termed mini-microplastic. This is a difficult task in the ocean due to their small size and irregularity. Here, we took advantage of the well-documented autofluorescence of many plastics (Langhals et al. 2015) and modified an epifluorescence microscopy approach normally used to enumerate planktonic microorganisms (Kemp et al. 1993; Taylor et al. 2012), to quantify oceanic mini-microplastics in surface seawater and salp gut contents. Specifically, we asked: What are the distribution and abundances of these mini-microplastics in surface seawater? Are salps ingesting mini-microplastics in situ? And, does the size distribution of ingested particles reflect that of available plastic particles?

Methods

Sample collection at sea

Surface seawater samples and salp specimens used in this analysis came from the following cruises: SEAPLEX (02–21 August 2009), R/V *Falkor* (21–30 October 2013), SKrillEx I (26–31 July 2014), and SKrillEx II (11–17 June 2015) (Supporting Information Fig. S1). Surface seawater samples (1–2 m) were collected in metal buckets, immediately filtered onto 5 μm pore polycarbonate filters, and frozen. We sorted salps from sodium borate-buffered 1.8% formaldehyde preserved plankton samples. These were collected via a 202 μm mesh bongo net at a tow depth of approximately 200 m or a surface-dwelling 333 μm mesh manta net. We tested for airborne plastic contamination during sample collection on a separate cruise in January 2017 by separately filtering both surface seawater samples and ultra-filtered Milli-Q water (*see* Supporting Information).

Salp identification and dissections

We sorted, measured, and identified salp species and life history stage from preserved specimens from each sampling location, located in three open ocean regions: North Pacific Subtropical Gyre (NPSG), California Current (CC), the transition region (TR), and a nearshore region. Life history stage was designated as blastozooid, the sexual chain-forming generation, or oozooid, the asexual solitary generation. Salp guts were dissected; however, any existing mucous nets and gill bars were not analyzed to avoid artifacts of net feeding (*see* Supporting Information).

Epifluorescence microscopy to identify plastics

Traditional epifluorescence microscopy techniques add fluorochromes to stain the DNA and proteins of plankton so that identifying features appear under different reflected wavelengths of light (Kemp et al. 1993; Taylor et al. 2012). Because our target was identification of plastics, not living organisms, we did not add any fluorochromes. We left prepared slides at room temperature for at least 24 h to diminish chlorophyll *a* autofluorescence of plankton before visualization. This ensured the most fluorescent particles on microscopy images

were likely microplastics, bacteria, or transparent exopolymeric particles (TEP) (Samo et al. 2008). We tested multiple plastic and nonplastic reference materials, such as cotton and wool, under the four light excitation channels of our microscope to determine their autofluorescence (Supporting Information - Table S1). Filtered surface seawater samples and salp gut contents were prepared for microscopy according to Freibott et al. (2014), using an all-glass filtration apparatus (*see* Supporting Information).

Enumeration of plastic and fiber particles

We created a decision tree to determine if a particle was plastic (Supporting Information Fig. S2). Generally, plastics appeared as long, thin fibers or flat fragments with sharp edges. Plastic particles fluoresced uniformly and did not have inner striations, coloration patterns, or features suggestive of biological particles, such as spines, nuclei, or organelles. Not all plastics fluoresce, so this was not used as a diagnostic feature (Supporting Information Table S1). Particles that were invisible under transmitted light but fluoresced under another light channel were determined to be TEP (Supporting Information Figs. S2, S3; Samo et al. 2008). Particles identified as likely diatom frustules, including chain-formers and pennates like *Pseudo-nitzschia*, were not counted as plastics. When in doubt, particles were not counted as plastic, so our estimates are conservative and most likely underestimate total mini-microplastic abundance.

Particles were categorized as short or long fibers and fragments (Supporting Information Figs. S5, S6). The lengths, widths, areas, and fluorescence were recorded for every fragment and short fiber (≤ 300 μm) in automated images. Long fibers (> 300 μm) were enumerated in separate, manual visual transects at lesser magnification to eliminate the possibility of double-counting single large fibers that were not visualized in their totality in automated images. Fibers < 200 μm in length were not counted in manual transects; however, there may be some overlap between the short fibers counted in automated images and long fibers counted in manual transects, due to the 200–300 μm overlap. We recorded long fiber color, length, and width with an ocular micrometer (*see* Supporting Information).

We analyzed plastic particles in filtered salp gut contents via epifluorescence microscopy. Because salp gut walls and ingested biogenic material can fluoresce, fluorescence was considered a secondary characteristic of ingested plastic over particle shape and reflectivity under transmitted light. However, fluorescence was checked to visualize inner striations or patterns characteristic of diatom chains. When in doubt, particles were not counted as plastic. The thick gut walls of salps and ingested biogenic material most likely occluded some plastic, so our data underestimate total plastic ingestion.

Ingestion rates

To calculate salp ingestion rates of plastic, mini-microplastic counts were divided by gut clearance times. We adapted the methods of Huskin et al. (2003) to calculate gut clearance times

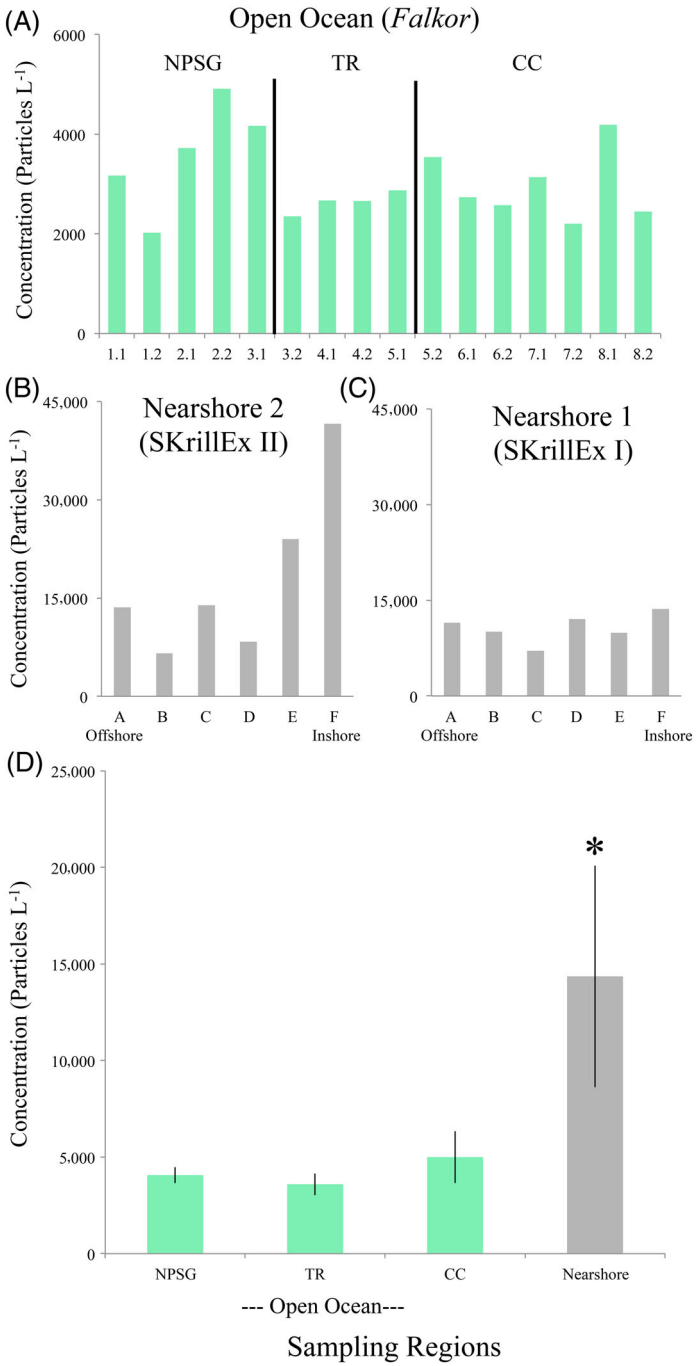

Fig. 1. Total plastic mini-microdebris concentrations, measured across the open ocean (Falkor; **A)**, and the nearshore (SKrillEx II; **B** and SKrillEx I; **C**). The mean concentrations from each sampling region, that is, the NPSG, the TR, the CC, and the nearshore were also compared (**D**). Small fibers and fragments were counted by digital image surveys, long fibers by visual counts. Green = open ocean (Falkor), gray = nearshore (SKrillEx I and SKrillEx II). (**A**–**C**) No significant spatial heterogeneity in concentrations within any one cruise ($p > 0.05$, Kruskal-Wallis). (**D**) Asterisk means nearshore samples are significantly different from all other regions ($p < 0.05$, Dunn's post hoc test with Benjamini-Hochberg adjustment).

for each species we identified, which ranged from 2.5 to 6.25 h (*see* Supporting Information).

Results

Epifluorescence and contamination

We found different patterns of fluorescence between plastic and biological materials, and when in doubt, particles were not counted as plastic. Using a controlled test, we determined that the vast majority of mini-microplastic materials in these filtered seawater samples were not from contamination during processing (Supporting Information Fig. S4).

Abundance and distribution of microplastics

Figure 1 combines all three plastic categories—short fibers, long fibers, and fragments—into total filtered plastic concentration at each station. We detected no significant spatial heterogeneity in seawater plastic concentrations across the *Falkor* transect (at 12 h intervals, Fig. 1A) for three open ocean regions: NPSG, CC, and the TR ($p > 0.05$, Kruskal–Wallis). Nearshore samples from SKrillEx I (Fig. 1B) and II (Fig. 1C) were collected at ~ 15 km intervals, and showed no significant spatial heterogeneity ($p > 0.05$, Kruskal–Wallis), possibly due to small sample sizes. Mean open ocean mini-microplastic concentrations compared to nearshore demonstrated significant heterogeneity between regions (Fig. 1D, $p < 0.001$, Kruskal–Wallis). Nearshore mini-microplastic concentrations differed from all other regions ($p < 0.05$, Dunn's post hoc test with Benjamini-Hochberg adjustment).

Open ocean mini-microplastic concentrations were on the order of 10^{2-3} L^{-1} for short fibers and fragments (Supporting Information Fig. S5A,B), with lower long fiber concentrations (10^{1-2} L^{-1}) (Supporting Information Fig. S5C, $p < 0.0001$, Kruskal–Wallis). In contrast, the fluorescent long fibers were 3.5–6.5 times more abundant than mean concentrations of nearshore short fibers and fragments on SKrillEx I (Supporting Information Fig. S6A–C), and almost eight times more abundant on SKrillEx II (Supporting Information Fig. S6D–F).

Almost every open ocean fragment and short fiber was < 333 μm in length and would have been missed by previous studies using larger mesh nets (Fig. 2). Long fibers were usually > 333 μm, but thin enough to easily slip through 333 μm mesh. The minimum lengths of fragments and short fibers were between 14 and 50 μm for all locations, approaching the 5-μm pore size of the filters. For long fibers, both surface area and length were significantly different among regions ($p < 0.01$, Kruskal–Wallis), with significantly shorter fibers in the TR ($p < 0.0001$, Dunn's post hoc test with Benjamini-Hochberg

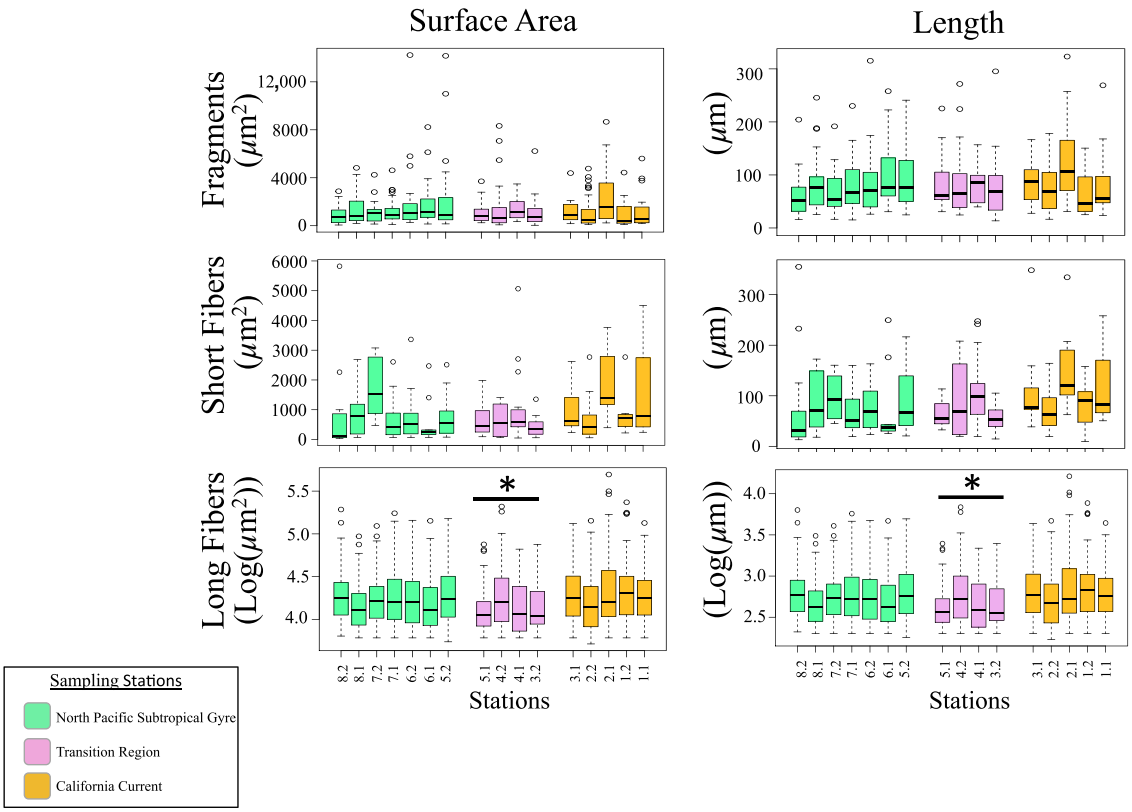

Fig. 2. Linear and areal dimensions of micro- and mini-microplastics sampled from sampling stations in the NPSG (green), the TR (purple), and the CC (yellow). The black line and asterisk indicate a region that is significantly different from the other two regions ($p < 0.01$, Kruskal–Wallis; $p < 0.001$, Dunn's post hoc test with Benjamini-Hochberg adjustment).

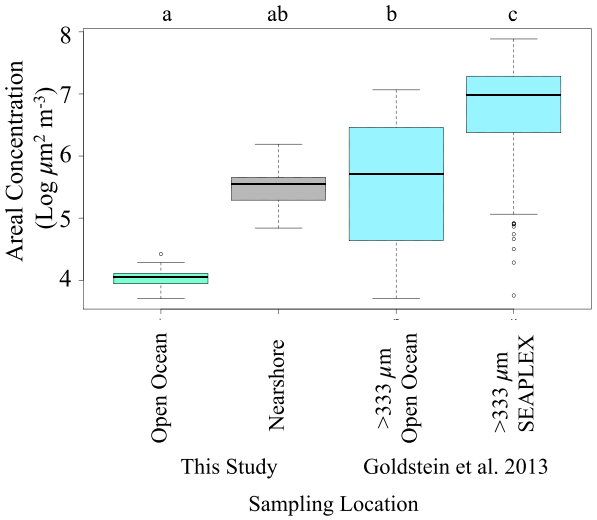

Fig. 3. A box and whisker plot, denoting the areal concentrations of plastics from the present study and Goldstein et al. (2013). Open ocean samples (green) represent the NPSG, TR, and CC combined, and Nearshore represents SKrillEx I and II samples combined. Circles are outliers beyond 3/2 outer quartiles. Goldstein et al. (2013) sampled plastics > 333 μm. This study's open ocean samples (a) and the > 333 μm open ocean samples from Goldstein et al. (2013) (b) do not differ significantly from nearshore samples (ab), although they do differ from each other. SEAPLEX concentrations (c) differ significantly from all other cruises ($p < 0.05$, Dunn's post hoc test with Benjamini-Hochberg adjustment).

adjustment). Similarly, in the nearshore samples (SKrillEx I and II), every measured fragment and short fiber length was < 333 μm (Supporting Information Figs. S7, S8). Spatial heterogeneity among stations is further discussed in the Supporting Information.

Individual particle surface area ranged from 3×10^{-5} to 0.71 mm^2, compared to Goldstein et al. (2013)'s findings, using a 333 μm net, which detected particles 0.01–565 mm^2. Goldstein et al. (2013) found plastic particle lengths ranging from 0.34 to 65.7 mm, while we found lengths from 0.01 to 16.27 mm (including long fibers). Ultimately, the most pronounced difference between our findings was not the size range of particles, but rather their concentrations. Mini-microplastics in this study were five orders of magnitude more abundant than the > 333 μm microplastics from Goldstein et al. (2013). However, when concentration was multiplied by surface area (log μm^2 of plastic m^{-3} water), we found that the > 333 μm microplastics had significantly higher areal concentrations than the < 333 μm mini-microplastics (Fig. 3, $p < 0.0001$, Kruskal–Wallis).

Ingestion by salps

Every single salp gut analyzed contained plastic. Blastozooids had higher ingestion rates than oozooids. In general, nearshore salps were larger than open ocean salps (Fig. 4, Supporting Information Fig. S9). The CC salps were the

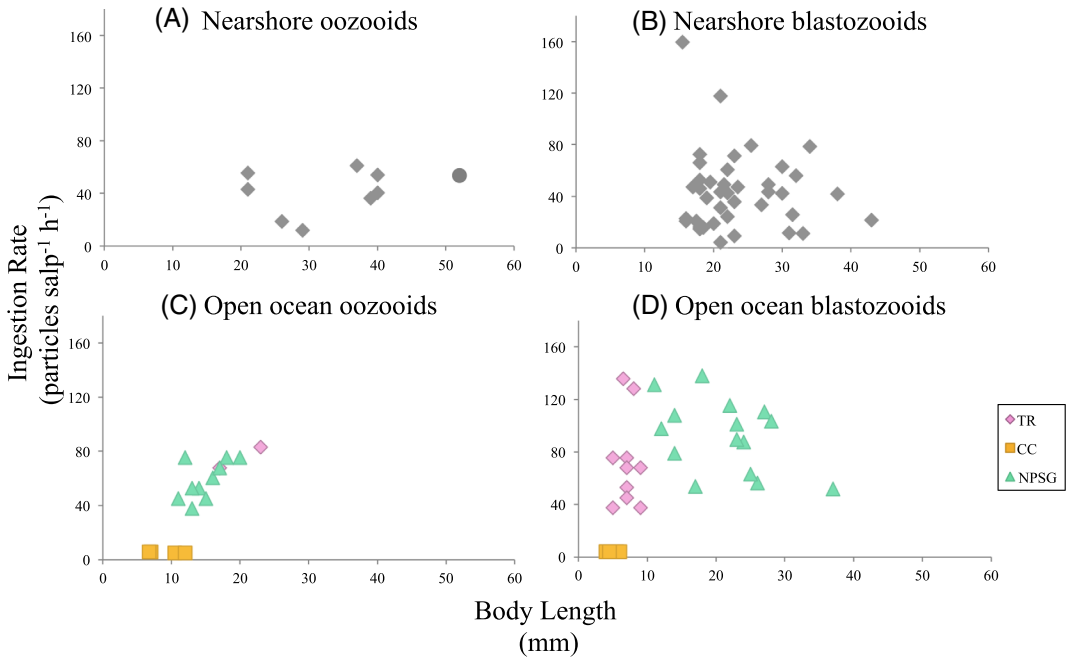

Fig. 4. Ingestion rate of microplastics plotted against body length of salps, obtained from epifluorescence microscopy of salp gut contents, and separated by salp life history stage and sampling region. For the nearshore oozoid (**A**) and blastozooid (**B**) salps, all species are *Cyclosalpa affinis* except one *Salpa aspera* (circle). For the open ocean oozoid (**C**) and blastozooid (**D**) salps, TR = transition region, CC = California Current, and NPSG = North Pacific Subtropical Gyre.

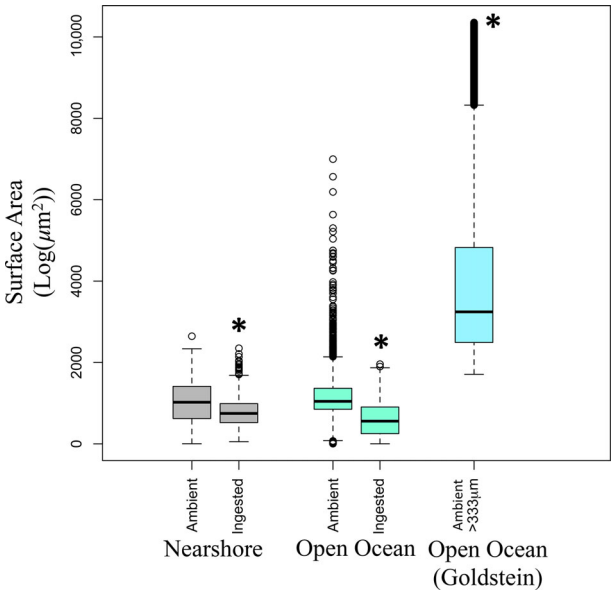

Fig. 5. Box and whisker plots of surface area of salp-ingested particles compared to surface area of ambient surface seawater particles. Gray: nearshore, measured in this study. Green: open ocean, measured in this study. Blue: microplastics > 333 μm, sampled in the open ocean (Goldstein et al. 2013). Circles are outliers beyond 3/2 outer quartiles. Asterisk indicates when a region is significantly different from all other regions ($p < 0.05$, Dunn's post hoc test with Benjamini-Hochberg adjustment).

smallest and had the lowest plastic ingestion rates (Fig. 4C,D). Excepting the NPSG and TR oozooids, there was no detectable relationship between body length and plastic ingestion rate for dissected salps. Although we found regional differences in mini-microplastic concentrations in the water column, there was no significant effect of region on salp plastic ingestion rate (Supporting Information Fig. S9, $p > 0.05$, Kruskal–Wallis). Fibers made up 91% of the total ingested particles. The surface area and lengths of fibers and fragments differed significantly between most regions (*see* Supporting Information).

We compared the size of ingested mini-microplastics with that of ambient mini-microplastics in surface seawater, both from our data and Goldstein et al. (2013) (Fig. 5). Most of the net-collected particles from Goldstein et al. (2013) fell within the size range of potential salp food particles (Vargas and Madin 2004). At all sample locations, the average size of particles consumed by salps was significantly smaller than the size of ambient seawater plastic (Fig. 5, $p < 0.05$, Dunn's post hoc test with Benjamini-Hochberg adjustment).

Discussion

We successfully used epifluorescence microscopy to identify mini-microplastic particles in natural seawater samples and salp gut contents. This method required careful judgment and expertise to distinguish biotic from plastic materials. Furthermore, autofluorescence of salp gut walls and biogenic materials made ingested plastic fluorescence only a secondary identification characteristic. This method allowed us to distinguish plastic from nonplastic particles and fluorescent from nonfluorescent plastic, but not to identify specific plastic types. Isolating plastic-type autofluorescence patterns under specific emission wavelengths may permit such differentiation in future work. However, our ultimate goal was to use standard epifluorescence microscopy techniques to differentiate plastics from nonplastic particles in order to obtain accurate bulk measurements of plastics < 333 μm, which the method accomplished.

This study may be one of the first to estimate the abundance of the smallest mini-microplastics in surface seawater, which are consistently under-sampled (Wang and Wang 2018). We found a mean plastic concentration across all locations of 8277 particles L^{-1} (8.3×10^6 particles m^{-3}). Our particle concentrations averaged 5–7 orders of magnitude higher than previous studies (Law et al. 2010, 2014; Goldstein et al. 2012; Wang and Wang 2018). This highlights the previously unquantified significance of mini-microplastics in marine debris counts.

Nearshore samples had higher plastic concentrations than open ocean samples. This agrees with published findings that have recorded similar spikes in plastic concentrations nearshore, close to populated areas, with a decline in plastic moving offshore (Law et al. 2010; Goldstein et al. 2012; Van Sebille et al. 2015). The difference in plastic concentrations between SKrillEx I and II may be explained by annual differences in rainfall and watershed input to these nearshore waters.

Many estimates of macro- and microdebris (Law et al. 2010, Goldstein et al. 2012, Van Sebille et al. 2015), including modeled debris trajectories (Maximenko et al. 2012; Eriksen et al. 2014), agree that the highest concentrations of open ocean marine debris occur in convergence zones of subtropical gyres. However, we did not detect a significant increase in mini-microplastic concentration in the NPSG and our open ocean samples were not significantly different across regions. Many possible sinks of mini-microplastics could account for this. Plastic below 5 μm in size presumably degrade beyond the detection limit of our method. Plastics can also be biofouled and sink out of surface water, or ingested and removed from the water. As plastic accumulates in the NPSG and breaks down into progressively smaller pieces, our data suggest that plastic < 333 μm is removed from the gyre through biofouling, ingestion, or degradation at the same rate it is being supplied. In the nearshore zone, however, mini-microplastics, especially long fibers (200 μm–17 mm range), likely have a higher rate of input than loss. All of these sources and sinks require further research to be better parameterized.

Goldstein et al. (2013) sampled almost no particles smaller than 0.333 mm × 0.333 mm (0.11 mm^2), due to the mesh size of the sample collection net, while we detected many particles below that limit (minimum size 3×10^{-5} mm^2). Our results show the majority of plastic concentrations occur between < 333 μm and 0.11 mm^2. Although the mini-microplastics we measured were more numerically abundant, they did not comprise the majority of the plastic surface area in the water. Organisms that colonize surface substrates in the ocean are

more likely to find surface area on micro- and macroplastics rather than mini-microplastics, despite the numerical abundance of mini-microplastics.

This is the first record of salp ingestion of microplastic in situ. Every salp dissected had plastic in its gut, regardless of species, life history stage, or region of the ocean sampled. Salp gut clearance times are on the order of 2–7 h (Huskin et al. 2003), so we are confident that by only analyzing the gut, we avoided artifacts of net feeding or other contamination. Airborne contamination is a major concern in modern microplastic work (GESAMP 2016), especially when samples are dominated by fibers, as in this study (91% of the salp-ingested particles). However, our processes of seawater filtration, slide preparation, and salp dissection limited contamination (*see* Supporting Information). Compared to filtered control samples, most of the plastics in our surface seawater samples were not contamination (Supporting Information Fig. S4).

We detected no regional differences in plastic ingestion by salps, excluding the much lower values of the CC salps. This finding is likely attributable to the very small body size of those salps. The CC salps had the lowest ingestion rate of any region, whereas for surface seawater, concentrations of mini-microplastics in the nearshore environment were significantly higher than the entire open ocean. Overall, however, both salp ingestion and surface seawater plastic concentrations had limited regional differences.

Salps are predominantly generalist suspension feeders (Vargas and Madin 2004) with ingestion based primarily on particle size, typically from < 1 m to 1 mm (Sutherland et al. 2010). All seawater mini-microplastic measured, and almost all the plastic in Goldstein et al. (2013), fall within their possible ingestion range. Yet, the salps sampled here ate significantly smaller pieces of plastic than were available in the ambient surface water. This may be explained by the fact that salps can efficiently collect down to submicron particles (Sutherland et al. 2010) and feed throughout a greater area of the water column than the surface, where larger, more buoyant plastic is retained.

Salps are of ecological importance due to several factors: their notoriously rapid growth and opportunistic reproductive rates that can lead to extremely high population densities or "blooms," higher filtration rates per individual than any other zooplankton grazer, and production of dense fecal pellets that can result in high vertical fluxes of this material to deeper depths (Madin and Deibel 1998). The large fecal pellets of salps have proven to possess rapid sinking and slow decomposition rates such that they can reach the deep ocean relatively intact (Caron et al. 1989), transporting organic carbon and potential microplastics with them. Our evidence for the widespread and universal consumption of microplastics by salps leads us to believe that salps may be an important vector of marine debris transport from the surface ocean to deep-sea communities. The transport of microplastics via salps may be critical to incorporate into microplastic export calculations as an overlooked output from surface waters.

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