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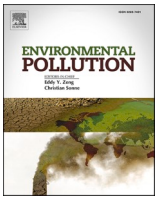

Foraging strategy influences the quantity of ingested micro- and nanoplastics in shorebirds☆

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ABSTRACT

Coastlines, including estuaries, mudflats, and beaches, are particularly susceptible to plastic pollution, which can accumulate from both marine and terrestrial sources. While numerous studies have confirmed the presence of microplastics (1–5 mm) along coastlines, few have focused on very small particles (<1 μm) or quantified exposure within the organisms that inhabit these areas, such as shorebirds. Here, we quantified small plastics (200 nm–70 μm) in two resident shorebird species in Tasmania, and compared this to quantities found in the surrounding sediments in order to investigate the potential exposure and transfer of particles within these ecosystems. Analysis was performed using a combination of flow cytometry for quantification of micro- and nanoplastics (200 nm–70 μm), and μm-FT-IR for validation and polymer identification of particles >5.5 × 5.5 μm. Micro- and nano-plastics were detected in 100% of guano samples from surface-feeding Eastern Hooded Plovers (*Thinornis cucullatus*) and 90% of Australian Pied Oystercatcher (*Haematopus longirostris)* guano, a species that forages for coastal invertebrates at 60–90 mm depth, and 100% of beach sediments. Hooded Plover guano contained 32 × more plastics, on average, than Pied Oystercatcher guano. Interestingly, the abundance of plastic particles within sediments collected from shorebird foraging sites did not appear to have a significant effect on the number of plastics the birds had ingested, suggesting the difference between species is likely a result of other variables, such as prey selection. The results of this study highlight the importance of including techniques that provide quantitative data on the abundance and size of the smallest possible particle sizes, and demonstrate the significant proportion of small plastics that are 'missed' using standard analysis tools.

1. Introduction

Increasing rates of plastic and chemical production have exceeded planetary boundaries putting the stability and future safety of the world's ecosystems (e.g., marine, terrestrial, and freshwater environments) at risk (Persson et al., 2022). Remarkably, the discovery of plastic in the ocean was first documented only a half a century ago (Carpenter et al., 1972; Carpenter and Smith Jr, 1972). Since then, an estimated 4.8–12.7 million metric tonnes of plastic have entered the ocean every year (Jambeck et al., 2015). While estimates of ~5 trillion plastic particles in the ocean's surface layer (top 10 cm) made global news in 2014 (Eriksen et al., 2014), this figure is now considered outdated with the true quantity of ocean plastics being much greater, but difficult to quantify due to differences in methodological approaches across studies (e.g., net/mesh sizes), ongoing fragmentation, and accelerating inputs (Watkins et al., 2021). For example, more recent analysis of data from the Great Pacific Garbage Patch estimated up to 3.6 trillion microplastics (>0.05 mm) could be floating in this one area (Lebreton et al., 2018). Much of this oceanic plastic will eventually make its way into estuaries and beaches where it can fragment via wave action and U.V exposure,

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leading to the accumulation of significant quantities (Browne et al., 2011; Vermeiren et al., 2016). As coastal bird species (e.g., shorebirds) rely heavily on these areas for foraging and breeding, they may be at higher risk of plastic exposure.

The definition of different particle size classes has also contributed to some of the confusion around just how much plastic is already present in the environment. In general, plastic particles <5 mm are typically defined as microplastics, but this definition has been the source of much disagreement among researchers (e.g., Gigault et al., 2018; Hartmann et al., 2019) with the size range often divided into additional categories, such as ultrafine (1 μm–1 mm; Provencher et al., 2017) and nano-plastics (1 nm–1 μm). Importantly, these smaller size classes are often difficult to detect in the environment (Oliveira and Almeida, 2019), raising questions around how much plastic exists within ecosystems.

Accurately defining particle size categories and choosing an appropriate range of sizes to record in our studies matter because nanoparticles exhibit different physical and chemical characteristics compared to larger particles, including microplastics. Changes in strength, conductivity and chemical reactivity of tiny plastic particles leads to increased bioavailability and ability to penetrate biological membranes (Gonçalves and Bebianno, 2021; Pelamatti et al., 2019). There is growing evidence both micro- and nanoparticles negatively impact biological processes in low (er) trophic level organisms, from bacteria to fish (Corami et al., 2022; Gonçalves and Bebianno, 2021; Mason et al., 2022). Changes in growth rates in marine plankton (Venâncio et al., 2019), significant decreases in cell viability in red microalgae (Gomes et al., 2020), and decreased fecundity in copepods (Cole et al., 2015) are adverse effects documented from the ingestion of small plastics. However, the effects of these particles in organisms at higher trophic levels (e.g., marine birds and mammals) are poorly understood, and it remains unclear whether ingestion of these small particles is associated with wildlife population decreases.

The ingestion of plastics by seabirds is well documented (Kühn and van Franeker, 2020) while comparable data for other avian groups, particularly shorebirds, is limited (Flemming et al., 2022; Hidalgo-Ruz et al., 2012; Lins-Silva et al., 2021; Lourenço et al., 2017; Rossi et al., 2019; Zhu et al., 2019). Shorebirds inhabit coastal ecosystems where micro- and nanoplastics are frequently recorded, and the birds play a critical role in these intertidal food-webs (Lourenço et al., 2017). Shorebirds can therefore act as bio-indicators for the health of sandy beach environments and provide valuable insights into the prevalence and effects of plastics within coastal ecosystems (Mathot et al., 2018; Ogden et al., 2014).

Necroscopies conducted on Australian shorebirds including Pacific Golden Plover (*Pluvialis fulva*), Bar Tailed Godwit (*Limosa lapponica*) and Sanderling (*Calidris alba*) found only one species, the Pied Oystercatcher (*Haematopus longirostris*) had ingested plastic (Roman et al., 2016). However, particle size (e.g., micro- or nanoplastic) was not specified by this study and the sample size was limited (n = 1 per species; Roman et al., 2016). A recent review of micro- and nanoplastic ingestion by shorebirds (i.e., debris recorded in the gastrointestinal tracts and/or guano) recorded 16 studies with data for 26 shorebird species spanning 10 countries (Flemming et al., 2022). These data suggest around 53% of the world's shorebirds are currently exposed to microplastics directly through sediment ingested in association with prey items, and indirectly through consumption of prey taxa that contain plastic. Overall, oystercatchers (Haematopodidae) had the highest incidence of ingested plastics (Flemming et al., 2022).

In Australia, research on microplastics in marine systems has been increasing; studies have documented levels in estuary, wetland and beach environments, and coastal seafloor sediments (Hayes et al., 2021; Lavers et al., 2019; Ling et al., 2017; Townsend et al., 2019). While most of the global research on microplastics in beaches has focused on macro-sizes due to visibility and simpler quantification methods, evidence suggests the vast majority of environmental plastics are micro- and nano-sized and can often be found buried in the sediment (Lavers and Bond, 2017; Lavers et al., 2019; Pohl et al., 2020). Overall, however, there is limited data on the prevalence and distribution of microplastics on Australian shorelines. Without such data, the availability and uptake by biota in these habitats cannot be quantified.

In this study, we identify and quantify plastic polymers ingested by two Australian resident shorebird species, the Australian Pied Oystercatcher and Eastern Hooded Plover (*Thinornis cucullatus)* using guano samples in order to gain insight into how shorebirds occupying the same habitat, but different feeding niches, may be differentially exposed to microplastic ingestion. This approach is minimally invasive (guano samples can often be collected with low disturbance) while also providing reliable data on the types and quantities of small particles being excreted by wildlife (Bourdages et al., 2021). Finally, we compare the type and size of particles with those recovered from coastal sediments collected from the birds' nesting territories in order to investigate micro- and nanoplastic exposure and how this relates to sediment depth.

2. Methods

2.1. Study sites

The study sites were selected based on knowledge of known breeding and roosting sites for both species whose populations have been surveyed for more than three decades. Tasmania's extensive coastline provides for extensive breeding habitat for both species, with an estimated 65% and 50% of the global populations of Eastern Hooded Plovers and Australian Pied Oystercatchers occurring in Tasmania (Woehler, 2021). All study sites were sandy beaches (Short, 2006).

2.2. Sample collection

Samples were collected from beaches and intertidal areas in southeastern Tasmania (Fig. S1; Table S1), within 100 km of metropolitan Hobart: South Arm Neck (Ralphs Bay), Goats Beach, Eaglehawk Neck, Safety Cove Beach, Orford Beach, Orford South Beach (south of the Prosser River), Rheban Beach and Saltworks Beach. The region supports numerous resident and migratory shorebird species, including the Eastern Hooded Plover which is listed as a Vulnerable species (BirdLife International,2022; Woehler, 2021).

These species have varied morphologies and foraging strategies; the Hooded Plover is small (90–100 g) with a short bill (17–19 mm; Marchant and Higgins, 1993b) while the Pied Oystercatcher (650–750 g) has a long bill (60–90 mm; Marchant and Higgins, 1993a). Sample collection commenced on October 1, 2021 and finished on November 29, 2021, once the shorebirds had established breeding territories and nesting sites for the 2021/22 breeding season.

A total of 30 guano samples was obtained, 15 from each of the two study species. For Hooded Plovers, the birds were observed using binoculars at a distance of approximately 20 m so that the deposition of guano was visible. Fresh guano samples were collected from the beach surface using a stainless-steel spatula and glass vial to limit the risk of contamination from plastics. Roosting Pied Oystercatchers were observed in groups of between 3 and 20 birds. Fresh guano samples were collected from roosting and foraging sites as described above and stored at −20 °C in a freezer, pending analysis at the Central Sciences Lab and Menzies Institute, University of Tasmania, Hobart.

Sediment samples were collected from each site within 2 m of where the sampled guano was deposited by the birds and foraging activity was observed. While most samples were obtained above the high tide mark, sampling spanned the entire width of the beach as this reflects the intertidal foraging range of the birds (i.e., from the water line to the vegetation; Colwell, 2010; Finn, 2010). A total of 18 sediment samples was obtained, nine from sediments associated with each of the two study species. Sediment was collected using a stainless-steel straw inserted into the sediment at two depths representing the average beak length of

each study species: 2 cm for Hooded Plover sites and 10 cm for the Pied Oystercatcher sites.

2.3. Plastic extraction from guano

Samples were allowed to thaw, placed into 50 ml tubes and the total wet mass of each sample was recorded. The removal of organic matter in the guano was performed using a digestion method, adding 15 ml of 11 M potassium hydroxide solution (KOH) into each tube, and allowing to sit overnight in a fume hood. The samples were then transferred to a heater and left to digest for a total of 5 day at 37 °C.

Post-digestion, 3 × 1 ml subsamples were pipetted into 15 ml tubes which underwent three initial washes with 12 ml of ethanol (100%) and 0.05 ml of hydrochloric acid (HCl, 37%) followed by three ethanol washes (resuspension 12 ml of 100% ethanol) in order to remove the KOH and prevent KOH precipitation reactions. After each wash, samples were centrifuged for 10 min at 3500 g.

2.4. Density separation

A high density 1.4 g/ml zinc chloride solution was prepared by dissolving zinc chloride salts into de-ionised water following recommendations outlined by Cole et al. (2015) who compared a range of different salts (e.g., ZnCl2 and NaCl) and concentrations, as well as their efficiency at separating plastics. Samples were then triturated in the remaining ethanol and transferred to a 2 ml tube (without adding extra liquid). The excess ethanol was evaporated using a desiccator for between 30 min and 1 h. The ZnCl2 solution was then added to each sample to separate the dense sediment and plastics. To mix the samples thoroughly and separate plastics from any sediment or leftover organic matter, samples underwent ten 1 s sonication pulses using an ultrasonic probe sonicator. They were left for 10 min for the density separation to take place, and the supernatant (containing the floating plastics) was then carefully pipetted and replaced back into the 15 ml tubes. After density separation, samples underwent a further three HCl washes to remove crystals. Each sample was topped up to 1.5 ml to allow for consistent concentration and volume for analysis and calculations. Polymers recovered are those with densities below the density of the solution being used. Those polymers not typically retrieved using this process include fluorinated plastics and some acrylic polymers. These are relatively rare plastics, and likely to sink in the ocean environment and thus not commonly end up on remote beaches or in surface-feeding birds.

2.5. Nile Red staining

The microplastics were stained using Nile Red to increase particle fluorescence for flow cytometry. For this, 10 μg/ml of Nile Red was dissolved in chloroform, as described in Tamminga et al. (2017) and 0.5 ml of the Nile Red solution was pipetted into each sample, and allowed to sit for 15 min for the Nile Red to stain the plastics. Lastly, five additional ethanol washes were completed to clean samples from $ZnCl_2$, HCl and any excess Nile Red.

2.6. Sediment samples

The sediment samples (n = 18) were first dried using a desiccator at −20 °C for 30 min, and approximately 5 g of dry sediment weighed out and placed into the same 50 ml tubes used for the guano samples. The samples were digested using KOH for a total of 48 h in the fume hood, as less organic material was present in these samples. Following digestion and ethanol washing, density separation was performed using zinc chloride, as per guano samples, and the supernatant removed and prepared for analysis.

2.7. μm-Fourier Transform Infrared spectroscopy (FT-IR) preparation

A total of 7 samples (2 of each species, 2 sediment, and a negative blank control) were randomly selected and underwent the same extraction methods described above. Nile Red was not added to these in order not to interfere with FT-IR analysis. Samples were filtered onto 25 mm Millipore anodisc filters with 0.2 μm pore size using a Prochem vacuum filtration set-up. After the sample was filtered, it was washed using a few drops of 100% ethanol, followed by a few drops of Milli-q water, and again with 100% ethanol to allow for quick dry. The filter was allowed to dry for 5 min with the vacuum left on, and then placed onto glass petri dishes, and covered with aluminum foil until analysis using μm-FT-IR.

2.8. Quality control and quality assurance (QA/QC)

Positive doped controls were generated through mechanically fractionation of collected marine environmental plastics of mixed plastic origin. The sizes of the microplastics generated were limited to <70 μm through filtration. Using FSC and SSC as size estimation methods, particles were found across the spectrum from 200 nm–70 μm. For each positive control, 1 ml was pipetted into a vial (in place of 1 ml of digested guano or sediment samples) and underwent the exact process as all other samples. Blank controls in which Milli-Q water were processed (including digestion) in identical fashion to the samples were run. The mean levels of microplastics observed in the blank controls were then subtracted from the samples, to estimate the microplastic levels in the samples. There was a total of six positive controls and six blanks (one each for each batch of samples). Additionally, a number of precautionary measures were adopted throughout the experiment in an attempt to reduce contamination. In the field, samples were collected and stored in glassware. In the laboratory, lab coats were worn at all times, and the preparation of samples were carefully handled with limited air exposure. In addition, all vials and pipettes were rinsed at least two times with deionised water prior to use. As a fume hood is designed to bring in air flow, rather than expel, and hence could potentially cause contamination, a fume hood was only used during handling of hazardous chemicals.

2.9. Data analysis: Flow cytometry

The retained micro- and nanoplastics were analysed using a Cytek® Aurora spectral flow cytometer (particle size reporting limit: 200 nm–70 μm), in combination with Nile Red staining. This allowed for precise measurement of particle abundance, down to approximately 200 nm, as well as approximate relative size distributions. All samples were pre-strained into fax tubes (using 250 μm strainer) prior to analysis, to protect the machines flow pathway from large particles. A volume of 25 μl was taken up by the flow cytometer immediately after straining, to avoid particle settling. All particles analysed during flow cytometry were characterised by their side scatter (SSC), forward scatter (FSC), violet fluorescence spectra (V2-A; violet excitation 405 nm, bandpass 420–453 nm and V15-A; excitation 405 nm, bandpass 765–795 nm) and yellow-green fluorescence spectra (YG4-A excitation 661 nm, bandpass 653–670 nm). Initial testing of blanks and microplastic doped samples stained with Nile Red identified a distinct plastic particle population using a V2-A and V15-A gate, followed by a YGA4-A gate (Fig. S2). This method requires both autofluorescence and Nile Red staining for detection and thus likely conservatively estimated the true microplastic burden due to these methods not robustly identifying all plastic types (Stanton et al., 2019; Wiggin and Holland, 2019).

2.10. Data analysis: micro-Fourier Transform Infrared spectroscopy (FT-IR)

μm-FT-IR was also performed to validate our flow cytometry findings

(i.e., a case study) and provide insight into the polymer types of the microplastics found within shorebird guano and sediments. Two of each species and two sediment samples were analysed using μm-FT-IR, as well as a positive doped control (as described in section 2.8), and a negative blank control sample.

The filtered samples on the Anodisc filters were analysed using a Bruker Hyperion 3000 microscope (Bruker Optics, Germany) attached to a Bruker Vertex 70 (Bruker Optics, Germany) spectrometer. The instrument and microscope compartment were flushed with dry air, at an airflow rate of approximately 200 L h-1 to ensure that carbon dioxide and water levels in the atmosphere remained consistent. The IR measurement was performed in transmission mode with a 15 × magnification objective and condenser. Measurements and analysis were conducted with OPUS 8.1 software (Bruker). A 64 × 64 FPA detector was used, with a binning of 2, resulting in 32 × 32 spectra per frame (dimensions of each frame approx. 176 × 176 μm^2). While the particle size detection limit for our equipment is 1 × 1 μm^2, we adopted a conservative pixel resolution (lower reporting limit) of 5.5 × 5.5 μm^2. Spectra were recorded with a spectral range from 3600 to 1250 cm^{-1}, four co-added scans, and a resolution of 8 cm^{-1}. For each sample, a new background measurement was recorded using the same parameters but with 128 co-added scans. Each filter was analysed by combining 31 × 31 FPA frames to create a hyperspectral image containing 984,064 spectra per filter.

The data from FT-IR was analysed using Purency software that was designed to classify plastic polymers using an automated machine learning algorithm and comprehensive built in spectral library of most common plastics. This produced an output of polymer type and count. Each classification of polymer is supported by a confidence value (relevance), and for this study we interpret a relevance value > 0.5 as a certainty that polymer has been identified correctly.

2.11. Statistical analysis

All statistical analyses were performed in R (version 4.03) and R studio (version 1.3.1; R Core Team, 2022). Differences in proportions were evaluated with the Fisher's exact test, and confidence intervals of proportions were calculated with Bayesian Jefferies intervals. Univariant linear models were utilized to assess the significance of the association between guano microplastics, sediment microplastics, and

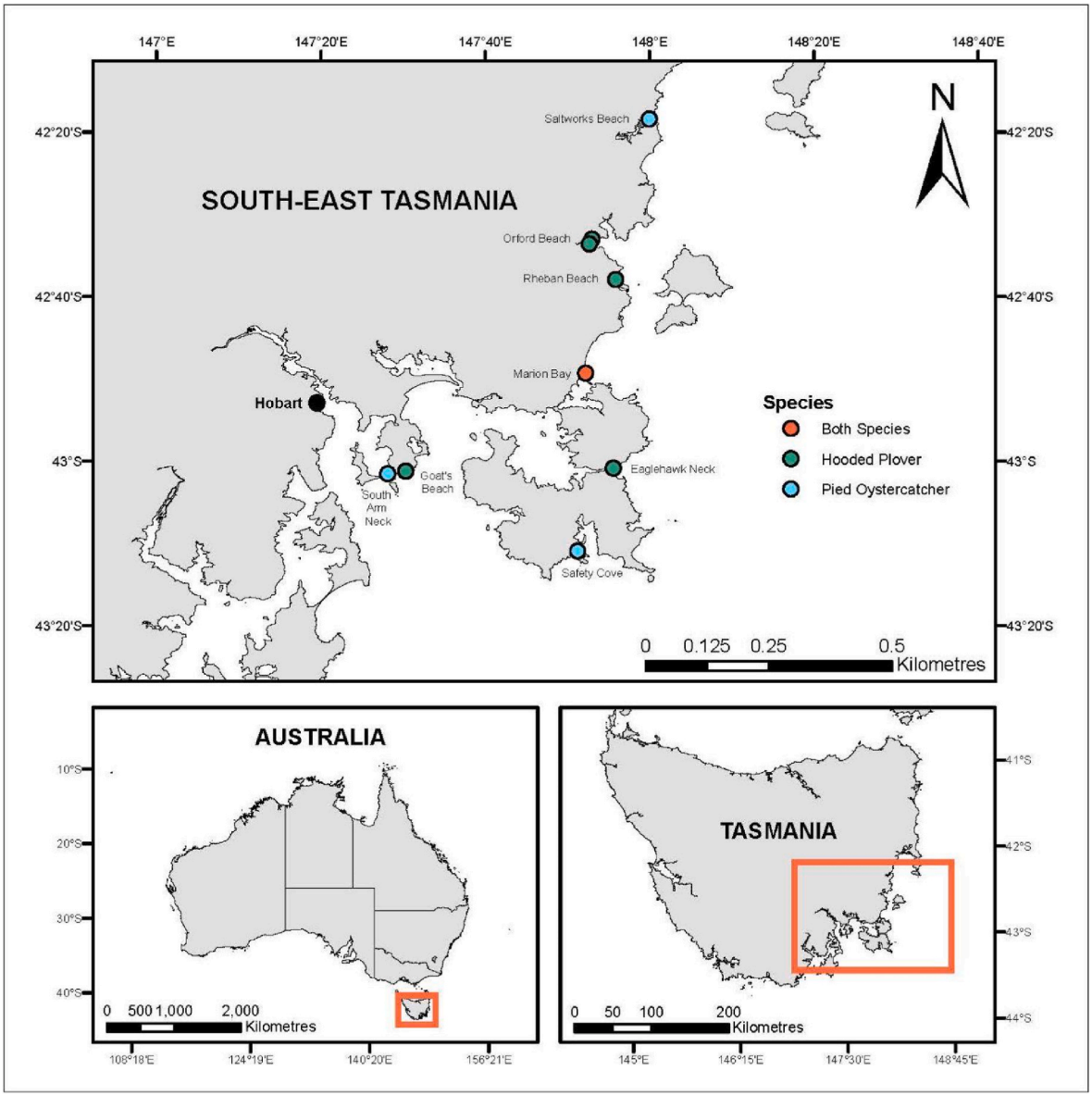

Fig. 1. Map of the sampling sites where both shorebird guano and sediments were collected in south-eastern Tasmania in Ogden et al., 2014.

species. To evaluate the significance of the effect of species on guano microplastics after adjusting for sediment microplastics a Type I sums of squares linear model was constructed with sediment microplastics as the first explanatory variable followed by species. Normality and homoscedasticity of the residuals were evaluated graphically with Q-Q plots and residual vs predicted plots, respectively. Box-Cox transformations were applied where necessary (Venables and Ripley, 2002). Flow cytometry analysis and gating was performed using the CytoExploreR package.

3. Results

3.1. Flow cytometry

Micro- and nanoplastics were found in 100% of Eastern Hooded Plover (n = 15) and 90% of Australian Pied Oystercatcher guano samples (n = 10). The mean concentrations for each species was 1678 ± 712.4 particles/mg and 77.93 ± 41.52 particles/mg wet weight, respectively. Overall, the mean number of microplastics found in guano samples (both species; n = 25) was 1038 ± 451.1 particles/mg. There was a significant difference in the number of microplastics in guano between species (p = 0.0016; Fig. 1Fig. 2A), with Hooded Plovers having significantly higher counts than Pied Oystercatchers.

All of the sediment samples (100%) collected on Tasmanian beaches were found to contain microplastics using flow cytometry, with a mean concentration of 48.01 ± 8.95 particles/mg from all 10 sites. Sediment samples collected at 2 cm depth (i.e., Hooded Plover guano collection sites) had higher concentrations (mean 65.86 ± 18.93 particles/mg) than samples collected at 10 cm depth (i.e., Pied Oystercatcher sites) with an average of 16.73 ± 1.35 particles/mg, however this difference was not significant (p > 0.05). Sediment was not found to be an influencing variable on the number of microplastics found in the guano for either species (p > 0.05; Fig. 2B). After adjusting for the effect of levels of microplastics in the sediment, the difference in microplastic numbers between species remained significant (r = 0.2293, p = 0.015; Fig. 3B).

3.2. μm-FT-IR

A total of seven samples (2 Hooded Plover, 2 Australian Pied-Oystercatcher, 2 sediment and one blank) were analysed using μm-FT-IR. Polypropylene (PP) was the most abundant polymer type identified using μm-FT-IR for both bird species and sediment (>80% for all samples), with a small proportion of polyethylene (PE), polystyrene (PS) and polyamide (PA; Table 1). PE was found in all guano samples, but not in either of the sediment samples (plover and oystercatcher collection sites). PS was found in the Rheban Beach sediment sample, and the Safety Cove oystercatcher guano. PA was found only in the Hooded Plover guano collected from Orford South Beach. A single particle of Ethylene-vinyl alcohol copolymer (EVOH) was found in the Rheban Beach sample. One of the sediment samples collected from Safety Cove contained no plastic particles. The blank sample also showed zero plastic particles.

4. Discussion

This study found the Hooded Plover and Australian Pied Oystercatcher are exposed to high amounts of micro- and nanoplastics within their coastal territories, with 24 of the 25 guano samples containing plastics. Overall, Hooded Plover guano contained significantly larger quantities of plastics compared to Pied Oystercatchers (Fig. 2).

During sample collection, Hooded Plover pairs were observed foraging in the upper limits of the beach, on dry sand, and within the dried wrack and small patches of vegetation. In contrast, oystercatchers foraged in more intertidal areas with wet sediment, including mud flats. Although the prey of these species would overlap to some degree, oystercatchers' probe deeper into sediment to catch prey, whereas the much smaller Hooded Plover (with shorter, narrower bill in comparison) forages in the surface layer of the beach sediments. These different foraging characteristics could explain, in part, the discrepancy between the ingestion of plastics.

Flow cytometry results found micro- and nano-plastics (200 nm–70 μm) in all sediment samples collected from 10 sites along the south-eastern Tasmanian coastline (Fig. 1). Marion Bay and Eaglehawk Neck Beaches had the highest quantities of plastics (mean 108.18 and 107.33 particles/mg, respectively) despite Eaglehawk Neck being the furthest from Tasmania's capital city, Hobart (Fig. 1). Orford South Beach had the lowest quantity of plastic (mean 9.65 particles/mg; Table S1). Sediments collected from shorebird foraging (guano-sampling) sites did not appear to have a significant effect on the number of microplastics the birds had ingested (Fig. 3A), suggesting the difference between species is likely a result of other variables, such as prey selection. This conclusion is supported by our analysis which adjusted for the microplastic counts found in sediments, with the resulting difference between the number of microplastics in each species remaining significant (Fig. 2B).

Sediments from numerous parts of the globe have shown high microplastic contamination within the top surface layer (Lavers et al., 2019; Torres and De-la-Torre, 2021; Veerasingam et al., 2021). The density of microplastics in beach sediments from Western Australia and

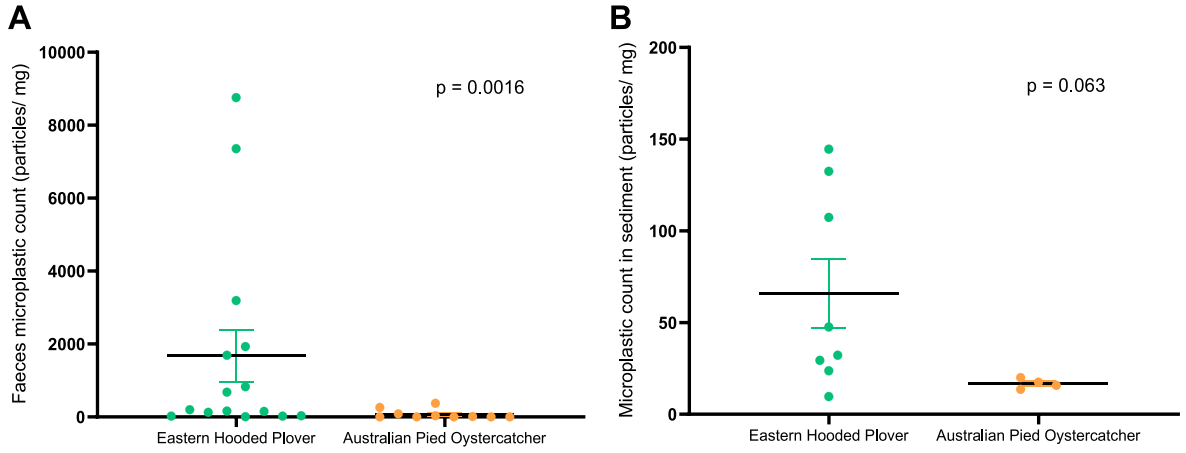

Fig. 2. The number of micro- and nanoplastics (200 nm–70 μm) in shorebird guano differed significantly between species, with Hooded Plovers having significantly higher counts than Pied Oystercatchers. **(A)** The concentration of plastics found in shorebird guano (n = 25, p = 0.0016; ordinary least squares using a Box Cox transformation). **(B)** The concentration of micro- and nanoplastics found in the sediment in close proximity to where guano was collected for oystercatchers and plovers, <0.05 (ordinary least squares lm using a BoxCox transformation). Bars represent mean ± standard error.

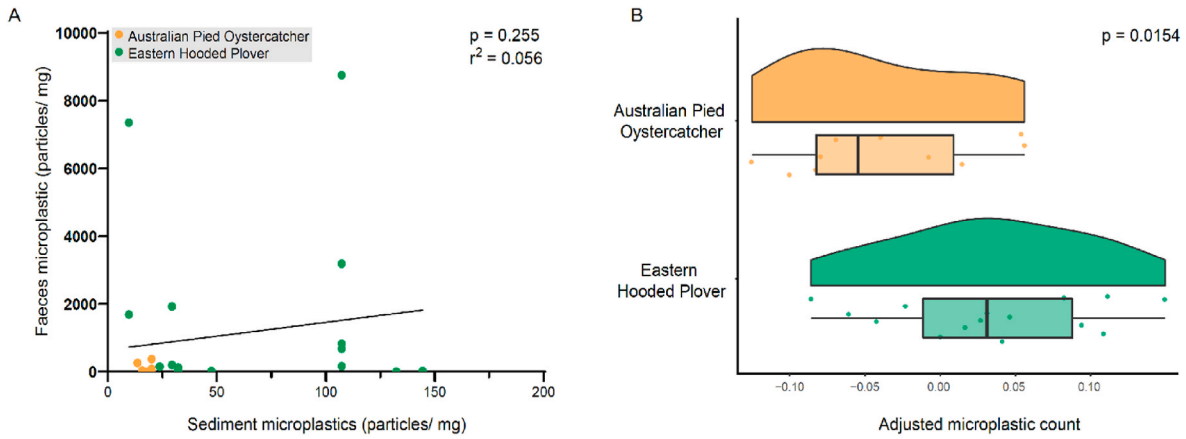

Fig. 3. The difference in guano micro- and nanoplastic (200 nm–70 μm) levels between species cannot be explained by the abundance of plastics in the sediment in which each species forages. **A)** Linear regression model showing concentrations of micro- and nanoplastics found in sediment plotted against concentrations of micro- and nanoplastics found in guano of Eastern Hooded Plovers and Australian Pied Oystercatcher (r = 0.056, p = 0.255). **B)** The difference in plastic counts is significantly different between species after adjusting for differences in sediment plastic. Type 1 linear regression, p = 0.0154, shown are raincloud plots and boxplots with median and interquartile ranges. For simplicity, only the term microplastic is written on the x-axis and y-axis.

Table 1
Micro- and nano-plastic abundance and polymer composition in guano and sediment (2 × Eastern Hooded Plover, 2 × Australian Pied Oystercatcher, 2 × sediments: Rheban Beach (representing foraging site of Hooded Plover) and Safety Cove (representing foraging site of Pied Oystercatcher), as identified using μm-FT-IR.

Polymer type	Pied Oystercatcher guano	Hooded Plover guano	Beach sediment from Pied Oystercatcher sites	Beach sediment from Hooded Plover sites
Polyamide (PA)	0	3	0	0
Polyethylene (PE)	4	1	0	0
Polypropylene (PP)	44	23	10	6
Polystyrene (PS)	1	0	0	2
Ethylene-vinyl alcohol (EVOH)	0	0	0	1
Polyvinyl chloride (PVC)	0	0	0	0
Polyethylene terephthalate (PET)	0	0	0	0

marine sediment cores from the United Kingdom decreased as sediment depth increased, with highest densities in beach sediments detected between 0 and 10 cm (Kukkola et al., 2022; Lavers et al., 2019). This corresponds with our findings, as the sediment collected at 2 cm depth had higher concentrations of micro- and nanoplastics at all Tasmanian sites compared with the sediment collected at 10 cm depth. This suggests Hooded Plovers might be consuming higher quantities of plastics as they forage along the beach surface and lends support to the idea that ingestion of plastics may be influenced by foraging strategy (i.e., beak length). In contrast, Lourenço et al. (2017) found the ingestion of microfibres differed among shorebird species, but was not related to observed foraging behaviours. As few other data are available, our understanding of precisely how morphology and foraging strategy among shorebirds influence plastic ingestion remains poorly described.

A shorebirds' prey can differ due to a number of variables (e.g., season, change in tides) which can affect prey assemblages. Prey and plastic availability can also depend on where along a beach shorebirds are foraging (Esiukova et al., 2021; Monk et al., 2020). Wrack lines on sandy beaches typically consist of seaweed and other debris, including plastics, washed ashore by tides, and this accumulation of detritus can heavily influence marine and terrestrial invertebrate assemblages (e.g., amphipods, isopods, and ants). Hooded Plovers are often observed foraging within wrack lines located along the sediment's surface (Butler et al., 2020; Schlacher et al., 2016), whereas Pied Oystercatchers tend to forage in the lower intertidal zones, and even in shallow water, capturing marine invertebrates buried within the sediment (Lauro and Nol, 1995; Taylor and Sarah, 2005). Although these species' habitats can sometimes overlap, this may contribute to each species' ingesting sediments from different regions of the beach and depths and potentially explain the significantly higher concentration of micro- and nanoplastics found in Hooded Plovers.

Micro- and nanoplastics in Tasmanian shorebird guano and adjacent sediments had similar polymer composition, with PP being the dominant polymer across all samples, followed by PE (Fig. 4D). These two polymers are the most commonly manufactured and widely distributed plastics worldwide (PlasticsEurope, 2021). Similar polymer compositions have been reported in coastal sediments in the Mediterranean, India (Frias et al., 2016; Karthik et al., 2018; Vianello et al., 2013), and now Tasmania, suggesting these polymers are likely ubiquitous in coastal areas. PP and PE are also positively buoyant in seawater (Hidalgo-Ruz et al., 2012), and are therefore likely to accumulate at the sea surface becoming widely dispersed and deposited on beaches and in estuaries. While most plastics such as PE, polyethylene terephthalate (PET), PS, polycarbonate, and PA6 have been shown to be resistant to KOH digestion, KOH can dissolve some polymers and this may explain the lack of detection in FT-IR, in particular cellulose acetate and polylactic acid (Kühn et al., 2017).

Polymer type, and other factors such as age, particle size, and environmental exposure, influence the chemical burden of plastics (Besson et al., 2020; Tanaka et al., 2019) and subsequent exposure of wildlife when items are consumed (Lavers and Bond, 2016; Lavers et al., 2014; Smith and Turner, 2020). In our study, PP and PE were the most abundant polymers in the guano of oystercatchers and plovers (Table 1). In contrast, most polymers in shorebird digestive tracts and guano from southern Europe and west Africa were polyacrylonitrile (PAN) and polyethylene terephthalate (PET; Lourenço et al., 2017). The discrepancy between sites could be due to temporal differences or the aims of each study, with Lourenço et al. (2017) focussing solely on microfibres. The similarity of polymer composition between Tasmanian shorebirds and sediments suggests accidental ingestion of polymers contained within sediments during foraging.

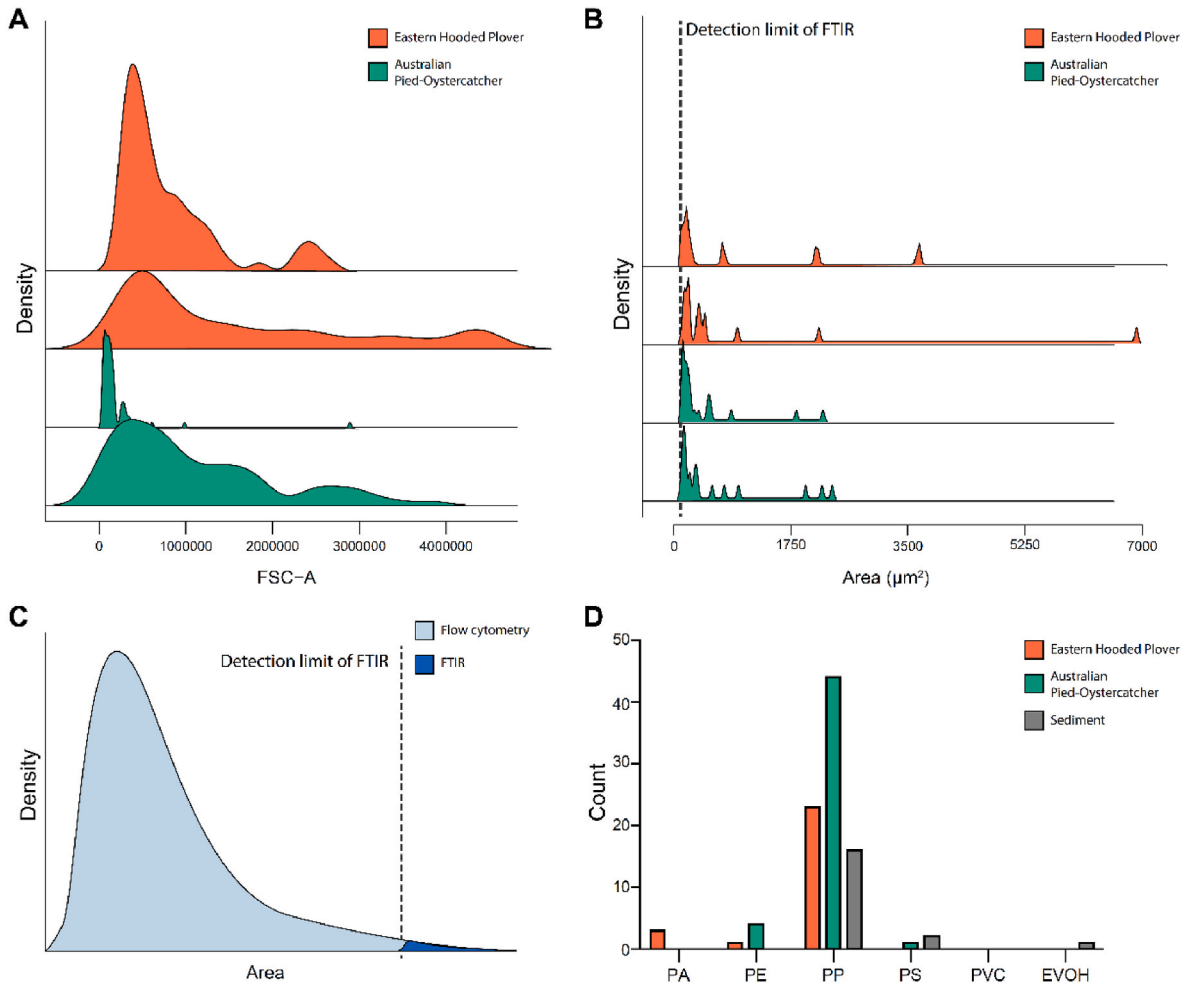

Fig. 4. Comparing the micro- and nanoplastics (200 nm–70 µm) profiles as identified by flow cytometry and Fourier Transform Infrared (FT-IR) spectroscopy of guano samples from Eastern Hooded Plover and Australian Pied Oystercatcher (n = 2). **A**) Density plots of plastic forward scatter (FSC-A) identified by flow cytometry. **B**) Density plots of the 2-dimensional area (μm^2) of the plastics identified by FT-IR spectroscopy. **C**) An overlay of the density plots of plastics size (approximated by FSC-A for flow cytometry) of the combined samples demonstrating the effects of the size detection limit of FT-IR spectroscopy on particle counts. **D**) Frequency of plastics of different polymer compositions between species and sediments, demonstrating the similarities in polymer profiles.

Concurrent analysis of the shorebird guano and sediment samples using both µm-FT-IR and flow cytometry provided an opportunity to demonstrate the benefits of each approach, and cross-validate data. Both FT-IR and flow cytometry analysis clearly indicate small-micro and nano-plastics are numerically abundant and prevalent in Tasmanian shorebirds and sediments (Table 1). Flow cytometry has a much smaller detection limit (minimum of 200 nm to a maximum of approximately 70 µm) compared with µm-FT-IR, thus flow cytometry identified three orders of magnitude more micro- and nano-plastics (Fig. 3C). Our data demonstrates a significant quantity of very small plastics are not recorded using FT-IR, a method that is more commonly employed in plastics studies. However, FT-IR remains an extremely useful tool for quantification of larger particles (minimum 1 × 1 μm^2), accurately estimating particle size, and is vital for polymer identification. Thus, FT-IR should be used whenever possible as a validation technique for methods which cannot identify polymers.

5. Conclusions

Plastics are persistent pollutants that will remain in the natural environment for the foreseeable future, posing an increasing number of serious and complex ecological challenges that already threaten the stability of Earth's systems (Lavers et al., 2022; Persson et al., 2022). Results of this study clearly indicate a significant number of very small plastics are frequently overlooked in the current literature for shorebirds, as well as seabirds (Keys et al., 2023). Technical limitations around identifying and quantifying nano-plastics in an efficient manner, including reliable detection of particles <5 µm using Nile Red and staining of non-polymers (i.e., Nile Red can also stain other types of lipophilic particles; Maes et al., 2017; Nel et al., 2021; Ruggero et al., 2020), will continue to pose a challenge in understanding the true abundance and effects these small particles are having on species and ecosystems, but when combined, flow cytometry and FT-IR provide a robust dataset on particle size (down to 200 nm), abundance, and polymer type in larger particles (>5 µm). Quantifying effects from exposure to these particles will require more data using these and other methodologies that can quantify plastics down to the nano-scale, which can then be applied within risk assessment and monitoring scenarios. This study has highlighted the benefits of combining multiple analysis tools for quantification of small plastics and has increased our understanding of just how pervasive the smallest particles already are in our environment.

Credit author statement

Karli A. Mylius: Funding acquisition, Investigation, Methodology, Writing – original draft. Jennifer L. Lavers: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review &

editing, Resources. Eric J. Wohler: Funding acquisition, Conceptualization, Resources, Writing – review & editing. Thomas Rodemann: Methodology, Resources, Writing – review & editing. Bianca C. Keys: Methodology, Funding acquisition. Jack Rivers-Auty: Formal analysis, Methodology, Resources, Supervision, Writing – review & editing.

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.

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Data availability

Data will be uploaded to the IMAS Data Portal upon the paper's acceptance and a URL link provided

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Appendix A. Supplementary data

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