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At second glance: The importance of strict quality control – A case study on microplastic in the Southern Ocean key species Antarctic krill, *Euphausia superba*

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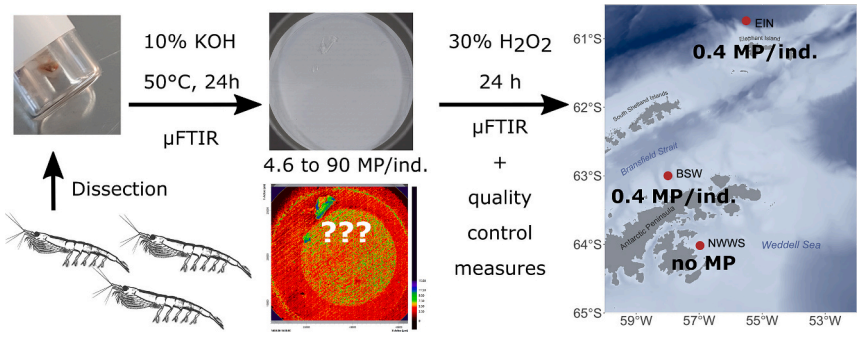
HIGHLIGHTS

- Analysis of pooled and individual krill samples for microplastic in three areas of the Southern Ocean
- Microplastics analysis using hyperspectral imaging Fourier-transform infrared spectroscopy (μFTIR)
- KOH digestion of krill formed a transparent residue on analytical filters which can be misidentified as microplastics.
- On average only 0.4 MP per individual krill specimen detected.
- Overestimation of microplastics pollution without strict quality control.

GRAPHICAL ABSTRACT



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ABSTRACT

The stomach content of 60 krill specimens from the Southern Ocean were analyzed for the presence of microplastic (MP), by testing different sample volumes, extraction approaches, and applying hyperspectral imaging Fourier-transform infrared spectroscopy (μFTIR). Strict quality control was applied on the generated results. A high load of residual materials in pooled samples hampered the analysis and avoided a reliable determination of

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putative MP particles. Individual krill stomachs displayed reliable results, however, only after re-treating the samples with hydrogen peroxide. Before this treatment, lipid rich residues of krill resulted in false assignments of polymer categories and hence, false high MP particle numbers. Finally, MP was identified in 4 stomachs out of 60, with only one MP particle per stomach. Our study highlights the importance of strict quality control to verify results before coming to a final decision on MP contamination in the environment to aid the establishment of suitable internationally standardized protocols for sampling and analysis of MP in organisms including their habitats in Southern Ocean and worldwide.

1. Introduction

In the last decades large numbers of plastic items have been released into the environment, and thereby have entered the world's oceans (Galloway et al., 2017). Slowly, these large items form smaller sized plastic particles (< 5 mm), microplastics (MP) by mechanical abrasion and weathering (Andrady, 2017). Even remote areas such as the Arctic and the Antarctic are already contaminated by MP (Waller et al., 2017; Peeken et al., 2018). Therefore, plastic pollution is nowadays of highest concern among scientists, the general public and policy makers worldwide. To provide valid information, standard operational procedures and internationally agreed standards are needed. This is still hampered by ongoing discussions about the optimal methods for the extraction and analysis of MP for this purpose (Lusher and Primpke, 2023; Primpke et al., 2023). Several initiatives tried to tackle this issue and among these, the EUROqCHARM project identified reproducible analytical pipelines (RAPs) and technical readiness levels (TRLs) for the analysis of MP for air, biota, sediment/soil and water samples (Aliani et al., 2023). For biota studies working on fish and bivalves samples (De Witte et al., 2023), it was found that mainly an alkaline digestion with potassium hydroxide (KOH) was performed for 58 % of the fish studies and 43 % of the bivalves studies. An oxidative treatment with hydrogen peroxide (H_2O_2) was performed in 24 % and 38 % of the studies for fish and bivalve samples, respectively. This was combined with a density separation in 16 % and 28 % of the studies for fish and bivalves, respectively (De Witte et al., 2023). Further, the Southern California Coastal Waters Research Project (SCCWRP) made a first assessment of standard operational protocols (SOPs) for the analysis of MP in different matrices covering biota (salmon), sediment and water ("clean" and "dirty") giving indications about suitable methods and issues for these methods (De Frond et al., 2022; Thornton Hampton et al., 2023).

The polar regions moved into the focus of MP research in the past years and high concentrations of MP in sea ice across the Arctic Ocean were reported, heightened the attention regarding MP pollution in polar ecosystems (Peeken et al., 2018). In addition, several studies also showed also evidence of MP in Arctic snow, surface waters and benthic organisms (Bergmann et al., 2022).

In contrast to the Arctic region (AMAP, 2021; Grøsvik et al., 2022; Kögel et al., 2022; Martin et al., 2022; Pollet et al., 2023; Primpke et al., 2023; Provencher et al., 2023) monitoring of MP in Antarctica starts to be discussed. Further, much less is known about the occurrence of MP in the Southern Ocean. Antarctica was thought to be a plastic-free, pristine habitat, since the Southern Ocean is a semi-enclosed system, which is encircled by the Antarctic Circumpolar Current (ACC) and its accompanying frontal systems (Gruber et al., 2019). This includes the Subtropical Front (STF), which represents the northern limit of the Southern Ocean, the Subantarctic Front (SAF), and the Antarctic Polar Front (APF, Constable et al., 2014). Therefore, MP contamination in the Southern Ocean has only received greater attention since 2017, when the first papers on MP occurrence in the region were published (Cincinelli et al., 2017; Waller et al., 2017).

Investigations of MP contamination of surface water samples from north and south of the Antarctic frontal system have confirmed the hypothesis that the ACC acts as a natural barrier for MP contamination from higher latitudes (Isobe et al., 2019). Water samples from regions south of the ACC were almost MP free compared to investigations of seawater samples in northern regions of the Front (Isobe et al., 2019; Suaria et al., 2020). This distribution implies local sources of MP in the Southern Ocean such as research stations, tourism, fishing, and research vessels (Cincinelli et al., 2017; Munari et al., 2017; Waller et al., 2017; Kanhai et al., 2018; Absher et al., 2019; Lacerda et al., 2019; Leistenschneider et al., 2021; Ergas et al., 2023; Huang et al., 2023; Jones-Williams et al., 2023; Kim et al., 2023; Mancuso et al., 2023).

How strong Antarctic organisms are affected by MP is difficult to evaluate (Bargagli and Rota, 2023). Only a limited number studies of studies have been published so far, which, unfortunately, differ greatly in the methods used to analyze MP, hampering a direct comparison of the results. Initial investigations on the scats of several Antarctic vertebrates, especially Gentoo Penguins (*Pygoscelis papua*), King Penguins (*Aptenodytes patagonicus*) and Antarctic fur seals (*Arctocephalus gazella*) have detected MP (Bessa et al., 2019; Le Guen et al., 2020). Investigations of 80 Antarctic gentoo penguin scats identified MP in 20 % of their scats, with a mean abundance of 0.23 ± 0.53 items per individual scats (Bessa et al., 2019). In contrast, a recent study on MP in the gizzard of Emperor Penguin (*Aptenodytes forsteri*) chicks showed no MP contaminations (Leistenschneider et al., 2022). Different studies found MP fibers in different Antarctic biota species. Absher et al. (2019) showed that zooplankton was observed to be entangled in MP fibers, but whether the sampled zooplankton ingested MP was not investigated. In *Harpagifer Antarcticus* and *Harpagifer bispinis* (Ergas et al., 2023) as well as in wild Antarctic whelk (*Neobuccinum eatoni*) (Bergami et al., 2023) different types of fibers made from cellulose and synthetic materials were found. In recent studies focusing on different fish species in Antarctica MP was found in fishes from 0.4 to 1.3 items per individual fish (Zhang et al., 2022; Geng et al., 2023; Mancuso et al., 2023).

Inspired by the study of Bessa et al. (2019) on MP in Gentoo Penguin scats, whose diet consists primarily of the Antarctic krill, *Euphausia superba* (hereafter termed krill), we aimed to study MP in the stomach content of freshly caught krill, in terms of MP particle sizes, numbers and chemical nature. In laboratory experiments, it has been demonstrated that krill can ingest MP (Dawson et al., 2018) and investigations on wild-caught krill are still limited (Wilkie Johnston et al., 2023; Zhu et al., 2023).

Krill is a keystone species in the Southern Ocean ecosystem with the largest biomass of a wild living animal on our planet, ranging between 300 and 500 million tons (Atkinson et al., 2009) This biomass corresponds to 300–400 trillion individuals (Bar-On et al., 2018). Each individual can grow up to 6 cm in length, has a fresh weight of approximately 1 g: and can live for up to 6 years (Tarling and Fielding, 2016). Krill serves as a direct trophic link between primary producers (mainly diatoms) and predators, accounting for up to 70 % of the food intake of predators such as seals, penguins, whales, squid and fish (Murphy et al., 2006). Krill tand salps are the key macro-zooplankton filter feeders in the Southern Ocean. Krill employs a feeding basket with mesh-size for catching nano- and micro-plankton in the water column (Boyd et al., 1984). During Antarctic summer when phytoplankton productivity is highest, krill-swarms, containing up to 10,000–30,000 individuals m^{-3} (Hamner et al., 1983) can graze down phytoplankton blooms within a few hours. Individual krill specimens have a filtration rate of up to 500 mL per hour (Morris et al., 1983; Boyd et al., 1984). Due to their large number and filtration capacity, krill can be seen as an ideal indicator species regarding MP contamination in the

Southern Ocean. Therefore, the presence of MP in the stomach content of krill would shed a new awareness on the marine Antarctic ecosystem, which is already affected by stressors, such as increasing temperature, ocean acidification, winter sea ice loss and increasing fishing effort.

So far, two studies recently published reported on MP in freshly caught krill (Wilkie Johnston et al., 2023; Zhu et al., 2023). While Zhu et al. (2023) identified 0.29 ± 0.14 and 0.20 ± 0.08 MP items per individual krill from the South Shetland Islands and South Orkney Islands, respectively, Wilkie Johnston et al. (2023) found 2.78 ± 0.56 and 1.55 ± 0.55 MP items per individual for juvenile and adult krill, respectively. Zhu et al. (2023) separated the chitinous outer shell of the krill, pooled the samples of the two stations and applied a treatment with KOH followed by a density separation with sodium iodide (NaI). In contrast, Wilkie Johnston et al. (2023) digested the complete krill specimen using a chemical-enzymatic treatment followed by a manual removing krill shells. After these steps further treatment using chitinase and a final density separation was performed. Due to large differences in the methods the reliable comparison of MP results is hampered.

To investigate the possible occurrence of MP in krill, a potential candidate for MP monitoring, we based our approach on alkaline KOH digestion of dissected krill stomachs. The experiments are designed to answer two questions, firstly, if pooling of samples is yielding similar results as individual samples, and secondly, if contamination by MP in krill is decreasing towards the Antarctic Peninsula. Here we analyzed different pool sizes and individual samples from three different regions in Antarctica using hyperspectral imaging Fourier-transform infrared spectroscopy (μFTIR; Primpke et al., 2020a). This is accompanied by strict quality control measures during sample preparation and analysis of the generated results (Primpke et al., 2023).

2. Material and methods

2.1. Sample collection

The krill samples were collected in the Southern Ocean near the Antarctica Peninsula, in the Bransfield Strait West (BSW), north of Elephant Island (EIN), and the northwestern Weddell Sea (NWWS) (Fig. 1, Table 1), on the Research Vessel Polarstern in austral autumn, during the expedition PS112, from the 18th of March to the 5th of May 2018. Krill sampling was performed using an Isaacs-Kidd Midwater trawl (IKMT) net, with a mesh size of 505 μm. The net was towed from 170 m depth to the surface. Freshly caught krill were measured and identified according to Makarov and Denys, 1981, fixed in liquid nitrogen and stored at −80 °C until further analyses at the Alfred Wegener Institute, Bremerhaven, Germany. From each sampling position, 20 krill were randomly selected for the analysis of MP in their stomach content.

2.2. Dissection of krill stomachs

For dissection of krill stomachs, the frozen krill was placed in a glass Petri dish connected to a cooling system (set to 1 °C) under a stereomicroscope (Leica S9i). The exoskeleton was removed from the cephalothorax region behind the eyes, where the stomach is located. The isolated stomach was placed in a pre-weighed glass vial and weighted on a Satorius balance (CP224S) with ±0.1 mg precision. Afterwards, individual stomachs were stored at −80 °C until further analysis for MP.

2.3. MP investigation of krill stomach content

The dissected krill stomachs from each station were processed in three sets (pool of 10 stomachs, pool of 5 stomachs, 5 individual stomachs, Table 1, Fig. S1), covering presumably different amounts of MP.

For each set per station, an initial alkaline digestion with KOH, commonly used for biota samples, was performed to denature the proteins and hydrolysis compounds in the guts (Kühn et al., 2015; Dehaut et al., 2016; Catarino et al., 2017; Lusher and Hernandez-Milian, 2018; Phuong et al., 2018; Zhang et al., 2022; Ergas et al., 2023; Geng et al., 2023).

Prior to the digestion process, the frozen stomachs per set were cut in half with a pre-cleaned Micro-Scalpell (Hammacher, 125 mm) and transferred to glass bottles with an NS 50/12 ground joint (Duran, Lenz

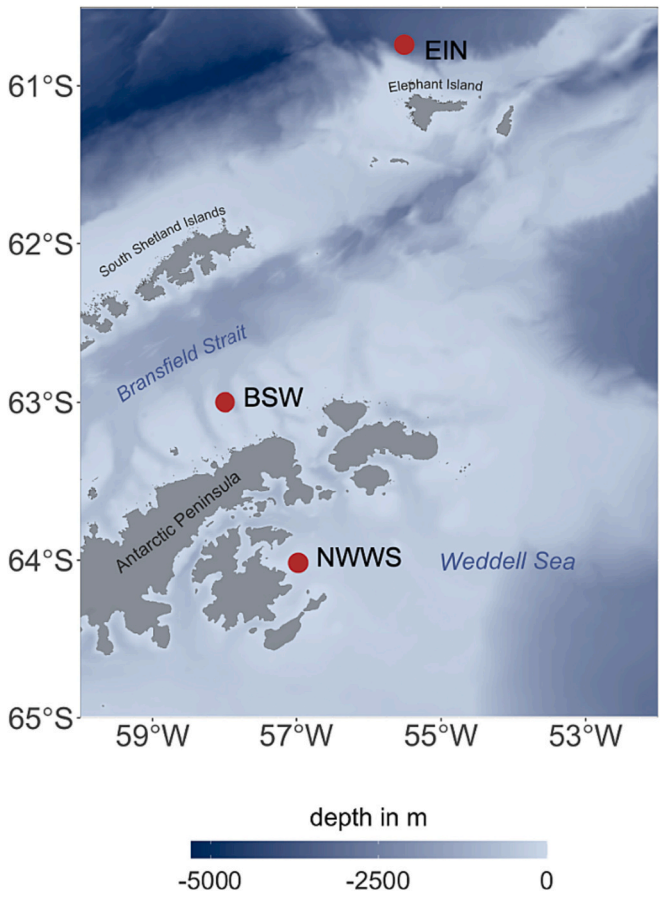


Fig. 1. Krill sampling positions. BSW: Bransfield Strait West; EIN: north of Elephant Island; NWWS: northwestern Weddell Sea.

Table 1
Name and position of sampling stations, stomach sets analyzed for microplastic, nomenclature of stomach sets.

Stations	Station Acronym	Position	Stomach sets analyzed	Nomenclature of stomach sets
Elephant Island North	EIN	Latitude: −60.73942, Longitude −55.5084	10 pooled stomachs 5 pooled stomachs 5 individual stomachs	EIN-10 EIN-5 EIN-1.1 EIN-1.2 EIN-1.3 EIN-1.4 EIN-1.5
Bransfield Strait West	BSW	Latitude: 62.9993 Longitude: −57.99357	10 pooled stomachs 5 pooled stomachs 5 individual stomachs	BSW-10 BSW-5 BSW-1.1 BSW-1.2 BSW-1.3 BSW-1.4 BSW-1.5
Northwestern Weddell Sea	NWWS	Latitude: 64.01753 Longitude: −56.97477	10 pooled stomachs 5 pooled stomachs 5 individual stomachs	NWWS-10 NWWS-5 NWWS-1.1 NWWS-1.2 NWWS-1.3 NWWS-1.4 NWWS-1.5

Laborglas GmbH & Co. KG, Wertheim, Germany) so that the stomach content can be released in KOH solution. The glass bottles with 10 stomachs (20 stomach halves) were filled with 20 mL of 10 % KOH solution, whereas the one with 5 stomachs (10 stomach halves) was filled with 10 mL of 10 % KOH. Five individual krill stomach halves were transferred into 5 mL glass culture tubes (50 × 14 mm, schuett-biotec GmbH, Göttingen, Germany), filled with 2 mL of 10 % KOH solution. Bottles and tubes with the krill stomachs were covered either by glass caps (glass bottles) or aluminum lids (glass tubes), gently mixed and incubated for 24 h at 50 °C in a dry oven (Memmert UN30). Each digestion approach of the pooled and individual krill stomachs was accompanied by a blank sample following the same procedure.

After incubation, the samples were neutralized using 20 % hydrogen chloride (HCl) solution (0.65 mL per 2 mL 10 % KOH solution) and finally transferred to polypropylene (PP)-supported aluminum oxide filters (Anodisc, 25 mm diameter, pore size: 0.2 µm, Whatman), using a custom-made filtration setup (see Fig. S2). Due to the design of the filtration device, the majority of the particles were located on a 10 mm in diameter area of the filter above the frit, with a 13 mm diameter filtration funnel used to avoid a particle ring formation at the filter funnel edge. Finally, the filters were rinsed with Milli-Q (pore size: 0,2 µm, Merck Millipore, Darmstadt, Germany) water and placed in glass Petri dishes within an activated silica gel desiccator cabinet for 48 h to dry. After drying all filters were subjected to µFTIR measurement (see µFTIR analysis section).

2.4. Additional treatment with H_2O_2

Due to obviously unexpected insufficient digestion results when using the alkaline digestion procedure outlined previously (see Results section), it was decided to re-treat the residual material of the stomach contents directly on filters using an additional oxidative treatment. For this purpose, after analysis with µFTIR, all filters of the alkaline digested individual stomachs were subjected to a treatment with H_2O_2. In contrast to the initial Anodisc filtration this was performed using the standard filtration system (Millipore Glass Microanalysis Filter Holder-Kit) to cover the whole sample with the peroxide solution similar to Peeken et al. (2018). The filter was placed onto the filter holder using stainless steel tweezers and the funnel afterwards carefully added on top. The joint between funnel, filter, and holder was sealed with parafilm and further secured with a clamp. The pre-filtered H_2O_2 (20 mL, 30 %, 0.22 µm GTTP, Carl Roth GmbH + Co. KG) was added onto the filter surface and soaked by applying vacuum (−200 mBar) for 30 s. Subsequently the filter was incubated unpressurized at room temperature for 24 h. The residual H_2O_2 solution was removed afterwards by applying vacuum and the filter washed carefully with Milli-Q water, and the samples dried for 48 h in a desiccator cabinet.

2.5. µFTIR analysis

In all cases the analysis for MP with imaging µFTIR was performed using a Hyperion 3000 µFTIR- microscope with a 64 × 64 focal plane array (FPA) detector (Bruker Optic GmbH, Ettlingen, Germany) connected to a Tensor 27 FTIR spectrometer (Bruker Optic GmbH, Ettlingen, Germany). The filters were placed on a CaF_2 (Calcium Fluoride) window (d = 25 mm × 2 mm thickness, Korth Kristalle GmbH, Germany) in a specialized sample holder. Prior to µFTIR measurement, a visual overview image was collected using a 4× visual objective.

The alkaline digested samples were analyzed using the 3.5× IR lens with a grid of 20 × 20 FPA fields recorded in transmission mode using a spectral range of 3600–1250 cm^{-1} (Löder et al., 2015), with a spectral resolution 8 cm^{-1}, a pixel size of 11.1 × 11.1 µm, Blackman-Harris 3–term apodization, a zero-filling factor of 2, and Power/No peak search for phase correction, following Andrade et al. (2020) and Cowger et al. (2020). The background was measured with 64 scans and the sample with 32 scans. The measurements were processed with the OPUS 7.5 software (Bruker Optic GmbH, Ettlingen, Germany). Due to an update to the instrument control unit the samples treated with H_2O_2 were measured using the same instrument and settings but processed with the OPUS 8.5 software (Bruker Optic GmbH, Ettlingen, Germany). Please note, that the different OPUS versions applied in this manuscript are mainly choose due to their compatibility with the used computer systems and data calculation speeds while having no effect on the measured or calculated data.

2.6. Data analysis

After measurement, the spectra data of the alkaline digested samples were analyzed using the automated analysis pipeline developed by Primpke et al. (2017) and the database from Primpke et al. (2018), using the Bruker OPUS 7.2 software (Bruker Optic GmbH, Ettlingen, Germany). Subsequently, image analysis of the derived results was performed using the automated particle analysis script developed by Primpke et al. (2017). For further quality control, after the data analysis selected spectra from the chemical images after data analysis were further investigated using the following commercial databases (Baseman polymer reference, Bruker Optics ATR-Polymer library (BPAD), Drug, Natural fiber library, Polymer, SR and Synthetic fibers ATR library) and Bruker OPUS 7.5 to identify the nature of the interfering compounds.

The peroxide treated samples were analyzed with siMPle v1.1.ß (Primpke et al., 2020b) using the siMPle database v1.0.1 (Primpke et al., 2018) and the extended database version from Roscher et al. (2022). Image analysis was performed using the enhanced image analysis script by (Kooi et al., 2021).

2.7. Quality control/quality assurance and contamination prevention

For quality assurance and quality control (QA/QC), general recommendation for MP measurements were applied (Primpke et al., 2023). Various measured were taken to avoid contamination by polymer particles and fibers from the ambient environment. Onboard the ship no particular contamination prevention was possible during the sampling, as the samples were collected during a larger krill sampling campaign. Due to the focus on the intact krill stomach, any external contamination from sampling was excluded, due to the self-contained nature of the organ.

In the laboratory, cotton lab coats were worn during all processes, with nitrile gloves worn for the dissection procedure and latex gloves for handling of chemicals. For the dissection, glass Petri dishes and stainless-steel dissection tools were used and cleaned with 70 % ethanol between each individual dissection. This took place in a laboratory with controlled room conditions. The dissected stomachs were individually stored in glass vials covered with aluminum foil lids, which had been previously oven dried at 200 °C for 4 h.

For the digestion processes all materials were conscientiously cleaned with ultrapure water (Milli-Q water) prior to usage. Furthermore, all chemical solutions (KOH, HCl and H_2O_2) were filtered over glass microfiber (GF/A) filters (pore size: 1.6 µm, GE Healthcare, Whatman) before use. Anodisc filters were cleaned with particle free pressurized air (Ballistol, Aham, Germany) to remove any residual particles from their packaging. The digestion processes were performed in a laminar flow cabinet (ScanLaf Fortuna, Lynge, Denmark) and all surfaces cleaned with ethanol before and after use. In the laboratories used for the digestion and µFTIR measurements, air cleaning systems (Dustbox®, Möcklinghoff Lufttechnik GmbH, Gelsenkirchen, Germany) with HEPA-14 filtration units were permanently active.

Due to the direct transfer of the digested stomach content from the glass vials to the Anodisc the samples were not spiked with known materials due to the known efficiency of the filtration step in various international inter-laboratory comparison (ILC) studies. As part of this procedure, the filtration funnel was always investigated for adhering

particles on the glass brim in contact with the filter.

All measured MP spectra were individually checked using the output file from the automated analysis via the "Load APA results" function of siMPle and ranked according to Primpke et al. (2017). All spectral assignments were additionally saved as image files.

3. Results

3.1. Krill stomachs

The krill stomach wet weight from the three sampling locations ranged from 9.7 to 772.3 mg, with an average wet weight per krill stomach (mean ± SE) of 267.6 ± 44.2 mg from the north of Elephant Island (EIN), 27.3 ± 2.5 mg from the station Bransfield Strait West (BSW) and 54.0 ± 5.6 mg from northwestern Weddell Sea (NWWS). The wide range of stomach weight reflects the different phytoplankton concentrations and thus the amount of food consumed by the krill caught in the respective regions. In the EIN region, a chlorophyll *a* concentration of up to 13 μg L^{-1} was measured, while in the other regions (BSW and NWWS) it was between 0.2 and 2.5 μg L^{-1}. The sampled krill from EIN were largest (47 mm ± 3.5 mm), followed by krill from the NWWS (39 mm ± 3.8 mm) and the BSW region (41 mm ± 3.5 mm).

3.2. Digestion/extraction of pooled and individual krill stomachs

In general, after alkaline digestion, all pooled stomach samples still displayed a high load of particulate material retained on the filters (filter cake, Fig. 2 B and D) which hampered μFTIR analysis (Fig. 2). Seemingly, this material consisted mainly of (undigested) chitin residues from the stomach tissue. As a consequence, these samples could not be further considered for MP analysis and it was decided to focus solely on samples representing individual stomachs. However, even for these samples some filters displayed a white or colored layer, indicating an insufficient digestion of the stomach contents and the presence of residual materials. This was further supported by an initial μFTIR analysis of the alkaline digested samples. In Fig. 3 a false color image of two filters is displayed, representing residues of individual krill stomachs from the northwestern Weddell Sea (NWWS-1.3) and Bransfield Strait West (BSW-1.2). Both filters seem to be covered by a layer of "rubber type 3" (RT3, Fig. 3 A) or "polyethylene chlorinated" type particles (PE-Cl, Fig. 3 B). Both polymer categories display strong peaks in the alkyl C—H stretch region (CH_2 asymmetric C—H stretch and CH_2 symmetric C-H-stretch), which are a general feature of alkane-based polymers but also of lipids and fatty acids. Together with the observation of the colored layer on the filter, the areal appearance of RT3 and PE-Cl rather than discrete individual particles suggests the presence of false-positive lipid or fatty acid residues and not the afore-mentioned polymers.

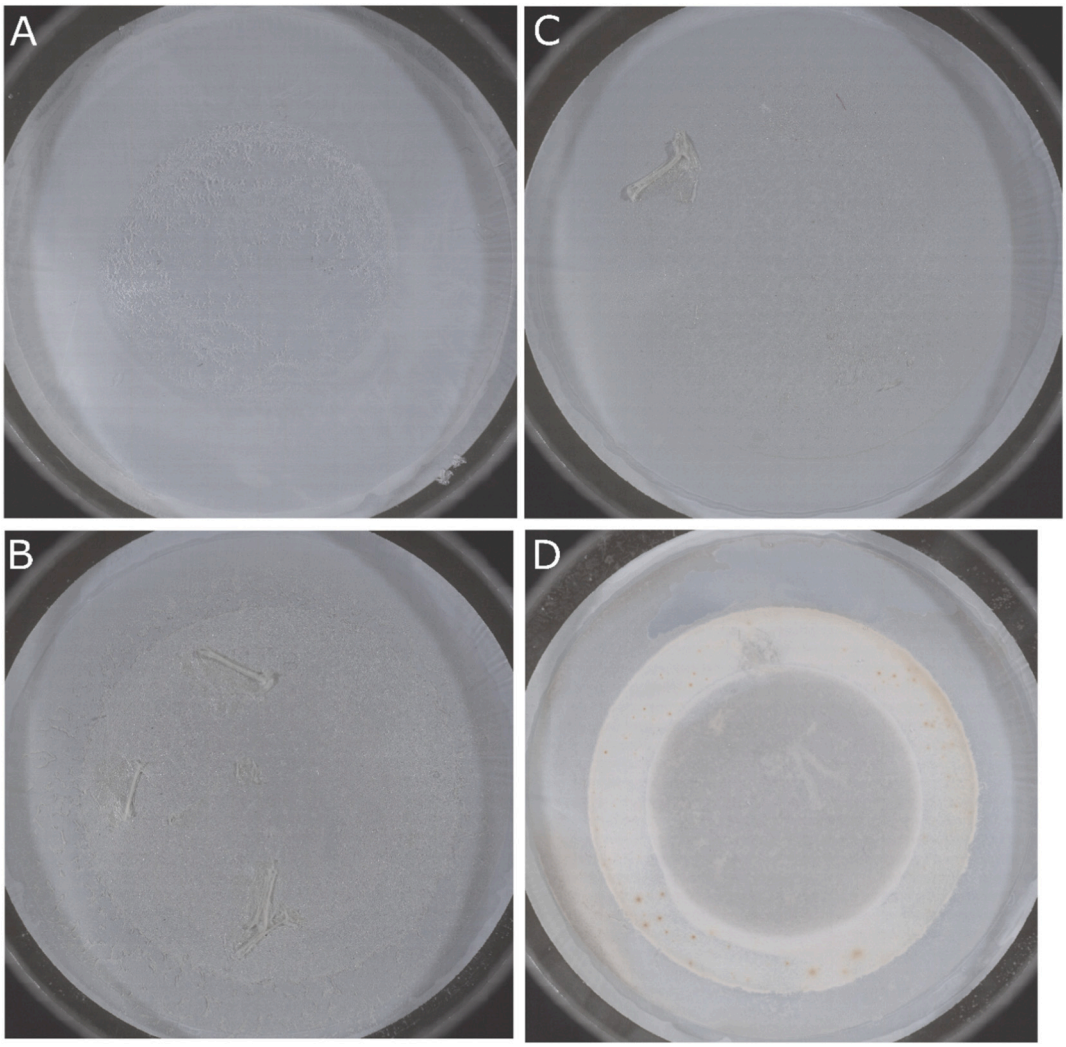


Fig. 2. Overview image of digested samples of krill stomachs from the north of Elephant Island on Anodisc: Blank filter of EIN-10 (A); Sample EIN-1.5 (B); Filter EIN-5 (C); Filter EIN-10 (D).

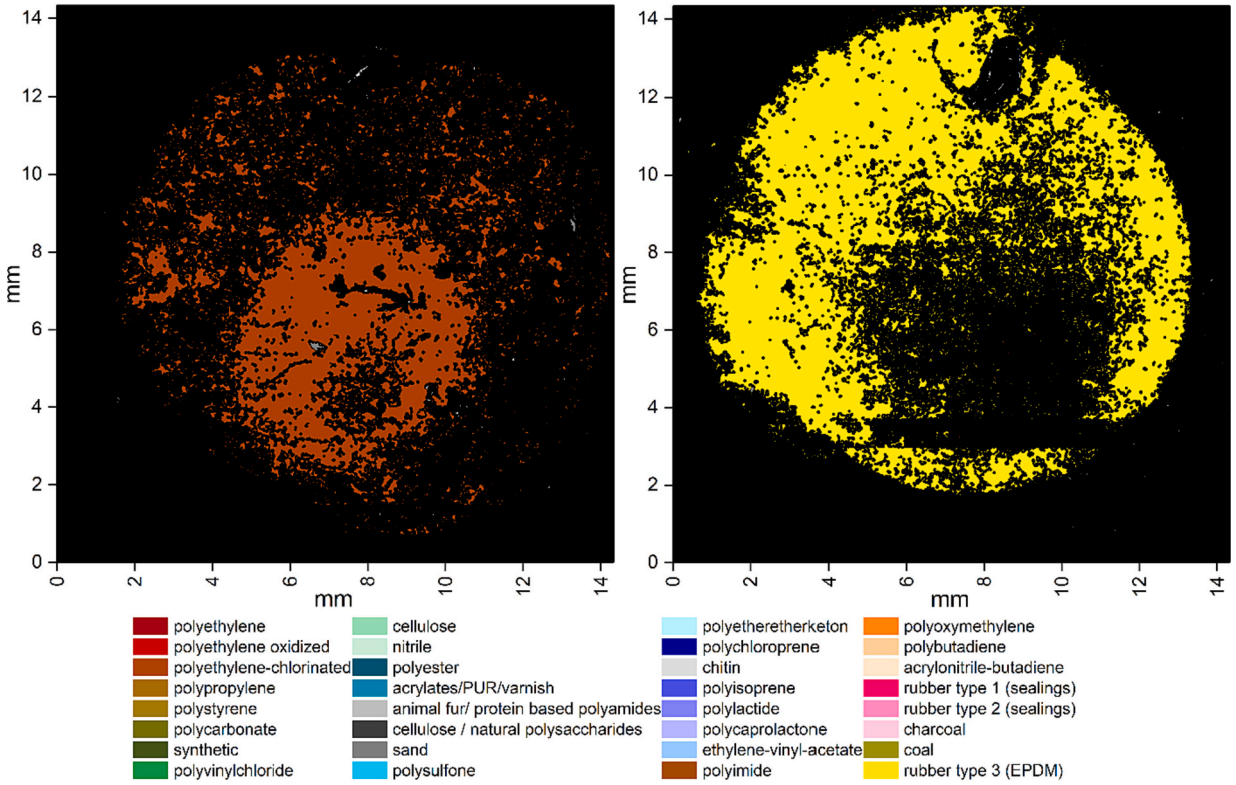


Fig. 3. Polymer dependent false color images for the samples NWWS-1.3 (A) and BSW-1.2 (B).

Still, other polymer types were identified in low numbers, such as polyamides (PA), acrylates/polyurethanes/varnish (APV), PP, rubber type 1 (RT1), rubber type 2 (RT2), polyethylene oxidized (PE-Ox), polycarbonate (PC), polychloroprene, polystyrene (PS), nitrile rubber (NBR), and polyester (PES).

Since the residual material which is displaying the strong signals in the alkyl C—H stretch region could not be removed by the initial alkaline digestion, it was decided to apply an additional oxidative treatment by using H_2O_2 directly on filters according to Peeken et al. (2018).

3.3. Digestion of the filters with individual stomachs with hydrogen peroxide

After treatment of the filters with H_2O_2, the signal intensity of the residual material was strongly reduced over the entire filter area. The stomach content from an individual sampled in the north of Elephant Island (EIN-1.1) is depicted in a false color image of the integral value for the peak of CH_2-bending vibration between 1480 and 1400 cm^{-1} for the alkaline treated sample (Fig. 4 A and C) and the H_2O_2 treated sample (Fig. 4 B and D). In both cases, the overall range of signal intensity was similar due to the presence of the PP-support ring of the Anodisc acting as internal standard (Fig. 4, A and B). By reducing the numerical range to 0–15 for the integral value to be considered, the differences between the alkaline treated sample (Fig. 4 C) and the post-processed filter (Fig. 4 D) were visualized. It was found that due to the decrease of residual material on the filter surface, following H_2O_2, the intensity of the peak between 1480 and 1400 cm^{-1} was reduced. The film-alike area was also reduced, from a strong white appearance towards dark grey or black, compared to the initial alkaline treated sample. During the subsequent data analysis, the number of assignments for PE-Cl and RT3 decreased for the measured filters while the number of assignments for PA, APV and polycaprolactone (PCL) increased to unexpectedly high levels (see Table 3 for an example). For pooled samples, it was found that the results could not be improved in a similar manner.

3.4. μFTIR analysis using an improved database

In recent years, the database for μFTIR analysis from Primpke et al. (2018) was improved by Roscher et al. (2022) and contains additional FTIR spectra of the lipid-rich cuticle of various plant leaves and different synthetic polyacrylamides. Initial results in Roscher et al. (2022) already indicated a reduction of misassignments for PE-Cl and RT3. Hence, this database was applied for the final data analysis of the H_2O_2 treated filters. In total, after the additional H_2O_2 treatment, reanalysis using the improved database the number of assignments to APV, RT3 and PE-Cl were extremely reduced in all investigated samples (see Table 2 for an example). For instance, in the stomach content of krill from the Elephant Island station 1.1 (EIN-1.1) and all samples from the northwestern Weddell Sea (NWWS) at first a high share of RT3 was detected. As final quality control a manual inspection of all assigned MP spectra was performed. It turned out, that the automated assignment of spectra to the RT3 and PE-Cl polymer categories leads to high false positive rates and both were excluded. Finally, after comparison with the blank samples, MP particles could be reliably identified in only four krill stomachs, being composed of NBR, PS APV and PP (Figs. S3 – S6). Two of the five krill stomachs from the Elephant Island station (EIN) and from Bransfield strait West (BSW) contained a single MP particle, whilst no MP particles were detected in stomachs from krill collected at the northwestern Weddell Sea (NWWS, see Table 3).

4. Discussion

Identification of MP in 4 out of 60 stomachs of *E. superba* after verification of results using methods such as μFTIR highlights the importance to apply strict quality control before interpreting microplastic contamination in the Southern Ocean. Moreover, investigations on whether pooling is a proper tool to investigate MP in krill showed this method to be hampered by the presence of residual materials on the filters. While sampling representing single stomachs could be analyzed by μFTIR (see for example Fig. 4), pooled samples (containing 10

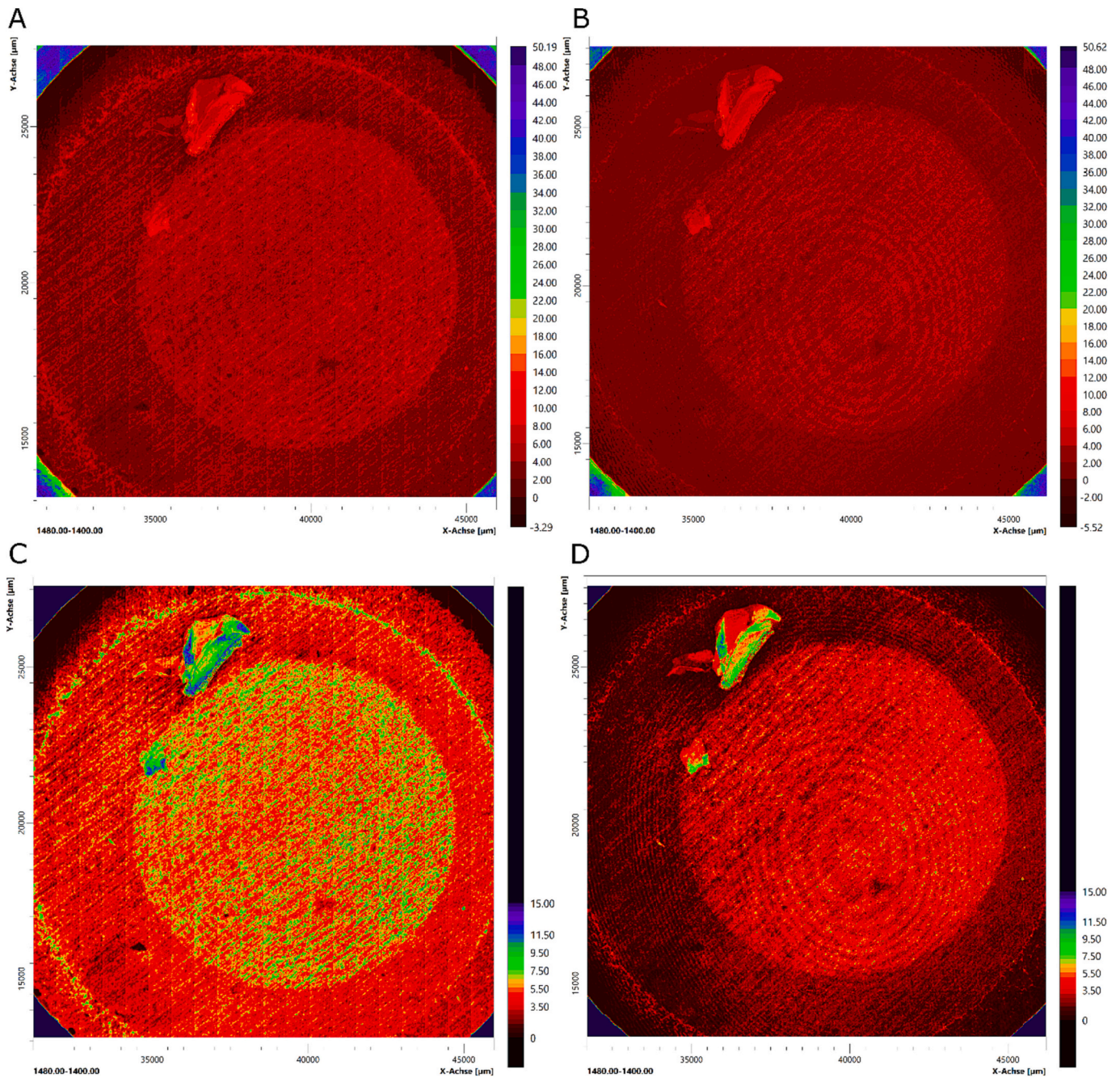


Fig. 4. Grayscale false color images of the filter of the individual alkaline digested krill stomach from the station Elephant Island North (EIN-1.1) prior to treatment with hydrogen peroxide (A, C) and afterwards (B, D) based on the integral of the CH_2 bending vibration between 1480 and 1400 cm^{-1}. The images A and B are scaled to the full determined range with a maximum around 50 at the polypropylene support ring while the images C and D are scaled for values between 0 (<0 black) and 15 (>15 white, mainly the polypropylene support ring).

Table 2
Example of sample EIN-1.3 for the overestimation of particle number derived after automated analysis for the different sample treatments and analysis databases. * Not assessed during quality control. PE: polyethylene; PE-ox: polyethylene-oxidized; PE-Cl: polyethylene-chlorinated; PA: polyamide; PES: polyester; APV: acrylates/polyurethanes/varnish; CE: cellulose; Chi: chitin; PCL: polycaprolactone; RT1: rubber type 1; RT3: rubber type 3; Cut: Cuticle.

Treatment	PE	PE-Ox	PE-Cl	PA	PES	APV	CE	Chi	PCL	RT1	RT3	Cut
Initial	20	4	3	7	1	0	33	1	0	1	9382	0
H_2O_2	0	0	140	38	0	641	109	14	10	0	1035	0
H_2O_2 + WWTP_DB	0	0	0	1	0	11	57	2	3	0	267	301
QA/QC	0	0	0	0	0	0	*	*	0	0	0	*

Table 3
Determined microplastic concentrations prior and after the application of further digestion steps and quality control measures.

Individual stomach	Microplastic concentration after alkaline extraction [N stomach^{-1}]	Average concentration after alkaline extraction [N stomach^{-1}]	Microplastic concentration after oxidative treatment and quality control [N stomach^{-1}]	Average concentration after oxidative treatment and quality control [N stomach^{-1}]
EIN-1.1	33	90.6 ± 67.1	0	0.4 ± 0.5
EIN-1.2	9		0	
EIN-1.3	358		0	
EIN-1.4	38		1	
EIN-1.5	15		1	
BSW-1.1	0	4.6 ± 2.3	0	0.4 ± 0.5
BSW-1.2	1		0	
BSW-1.3	13		1	
BSW-1.4	6		1	
BSW-1.5	3		0	
NWWS-1.1	135	45.6 ± 23.6	0	0
NWWS-1.2	9		0	
NWWS-1.3	9		0	
NWWS-1.4	25		0	
NWWS-1.5	50		0	

stomachs) were partly overloaded by the presence of chitinous residues. While analysis was clearly affected when 10 samples were pooled, even 5 stomachs in one pool occurred to cause an issue, although of lesser impact on the following analysis. Our study focused on having a minimum of interaction with the sample to avoid contamination. However, our findings suggest a further removal of stomach tissues and thorough rinsing as a solution for future studies using pooled samples. This approach was already successfully applied by removing cuticle residues from krill with tweezers when analyzing full Krill specimen (Wilkie Johnston et al., 2023).

Further, in our study we found that residues, likely from the lipid rich diet of krill, formed a film on the analytical filter surface, resulting in a false interpretation of the number of MP particles in individually analyzed krill stomachs (Table 3) and even more for the pooled samples. The false interpretation resulted in up to 90.6 MP particles on average per krill stomach (Table 3). It was found that the design of the extraction protocol is of high importance for the subsequent analysis. Due to the lipid rich diet of krill, the KOH digestion period of 24 h for individual stomachs still yielded fine residual precipitates in the solution. During filtration these particles form a thin film-alike layer of material on the filter surface as visualized in Figs. 2 and 3. To investigate the source material, individual spectra were selected and compared against commercial libraries. In Fig. 5, two examples are shown, with high assignment rates to the polymer types of PE-Cl (A) and RT3 (B) from the automated analysis.

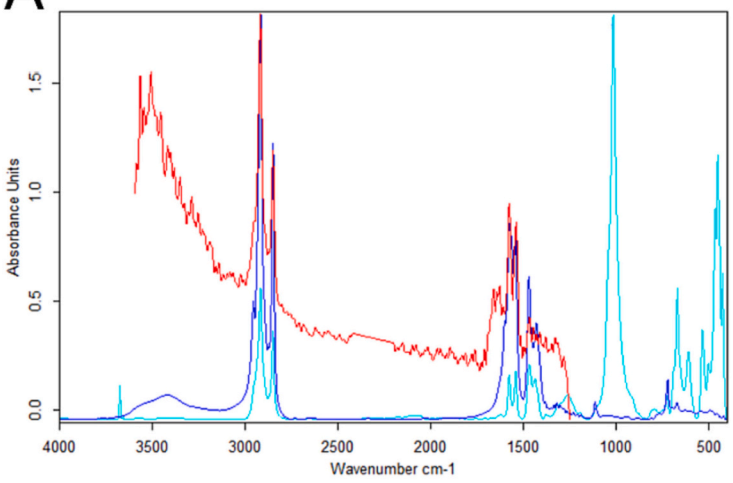
In both cases, database hits for stearic acid-based materials were found. This type of material is known in MP research to generate false-positives (Witzig et al., 2020) in all major analysis techniques. While Witzig et al. (2020) showed that nitrile rubber gloves release these materials, the KOH digestion and following neutralization yielded similar materials. While KOH digestion is one of the main applied chemicals for alkaline digestion for biota samples, only very limited information on interferences with FTIR measurements has so far. Wagner et al., 2017 mainly indicated the presence of residual peaks in samples from gastrointestinal tracts of fishes but did not discuss issue on the analytical results. Other studies working on lipid rich samples report from issues prior to the filtration either solved by adding surfactant (Hove et al., 2023; Lopes et al., 2022), different extraction methods (Jaafar et al., 2020), or the use of coconut oil (Kim and Hwang, 2023).

Additionally, due to the formation of a thin layer on the filter surface, covering any actual particles present, the measured μFTIR spectra will consist of mixed signals from the overlying stearate material and other residual materials, such as the chitinous stomach walls. This situation poses a high risk of false-positive interpretation for measured spectra, independent of the type of analysis applied (i.e. μFTIR, Quantum Cascade Laser-Infrared (QCL-IR) or Laser Direct Infrared (LDIR); Primpke et al., 2023). Further, until such a material is not securely removed, a pooling of krill stomachs cannot be recommended using KOH as initial digestion agent as these forms even thicker films (see Fig. 2).

Whilst data treatment processes, like spectral subtraction or component analysis, are available to separate the stearate spectra from the measured data, the high similarity to many plastic polymer spectra in the database hampered such a data processing step during the reanalysis in our case. In contrast, following the treatment with an oxidative digestion using hydrogen peroxide (Peeken et al., 2018), it was found that the general signal intensity (Fig. 4) and number of assignments for PE-Cl and RT3 decreased, indicating a good success for the performed treatment. Additionally, the number of other polymer types like APV, PA and PCL increased as the filter surface was oxidized. Due to this process the ratio between carbon-oxygen and carbon-hydrogen bonds was reduced, and higher assignment rates towards database entries with containing these two types of bonds were found. This situation could be overcome by the application of an improved database, containing more naturally lipid-rich materials (i.e. plant cuticle) and diversity of polymers (polyacrylamide; Roscher et al., 2022). Its application significantly reduced the number of assignments to different polymer types, allowing a detailed quality control on the assigned spectra.

Comparing with other studies, we mainly found MP particles of synthetic origin while Zhu et al. (2023) mainly identified synthetic MP fibers and Wilkie Johnston et al. (2023) a majority of MP fibers over MP fragments. The fibers identified in our samples and blank measurements consisted of chitinous or cellulose materials. Interestingly, the recently published studies by Zhu et al. (2023) and Wilkie Johnston et al. (2023) on MP in Antarctic krill showed similar polymer types as those initially measured from krill samples in our study (Table 2). As in our study, the material from the digestion process was directly transferred to the final analysis filters. This indicates that the authors may had unknowingly the same issue as presented in our study. While one study states that FTIR was applied for their analysis, no further details of the spectral library as recommended by Cowger et al. (2020), measured spectra or images of the identified particles were presented, preventing a cross-study evaluation. In contrast to this, the other study applied the same database and software as our study for the analysis of peroxide treated samples (see Table 2). In both cases, the same materials were found like the natural materials like cellulose, chitin and natural polyamide but also synthetic material like APV, PE-Cl, PA and ethylene-vinyl-acetate. Only due to the assessment of the full filter area compared to an optical preselection by Wilkie Johnston et al. (2023) it was possible in our study to get aware of the film-alike situation on the filter surface. The direct filtration to analytical filters is not specific for Antarctic Krill and was also applied for fish samples of the same region using KOH digestion (Zhang et al.,

A



Substanz	polyethylene_chlorinated_42%
Kurzzeichen	polyethylene_chlorinated_42%
Hersteller	Scientific Polymer Products, Inc.
Nummer	186
Form (Pulver, Pellet, Folie, Stü	powder
Farbe	white
Messmethode	ATR
Entry No.	214
Library name	BASEMANN_POLYMER_REFERENCE_DATABAS
Library description	Database containing spectra from polymers a

Color	Hit Quality	Compound name	CAS Number	Molecular formula	Molecular weight
	618	OCTADECANOIC ACID, ION(1-) CALCIUM-STEARATE, SALT		C18H35Ca1O2	323.225
	553	polyethylene_chlorinated_42%			

Color	File	Path	Spectrum Type
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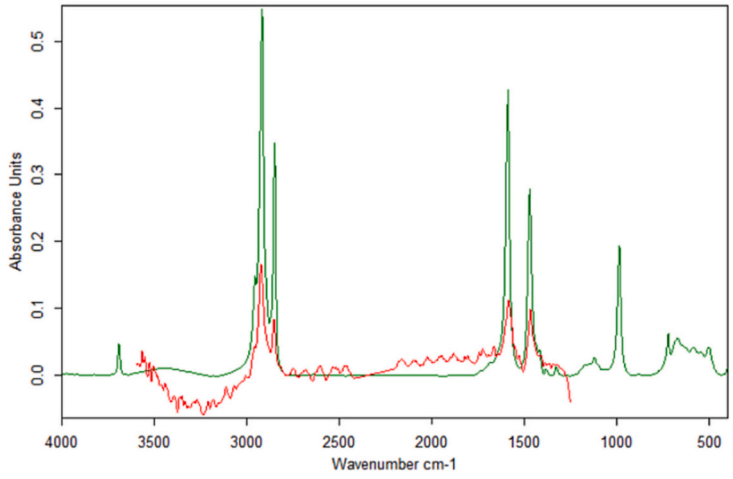
B



Compound Name	STEARIC ACID, ALUMINIUM SALT * OCTADE
Molecular Formula	C18.H35.AL1.O2.+2.
Molecular Weight	310.244
CAS Number	
Registry Number	19483
Entry No.	2573
Library name	BASF.S01
Library description	BASF
Copyright	Copyright 1991 Bruker Analytische Messtechn

Color	Hit Quality	Compound name	CAS Number	Molecular formula	Molecular weight
	527	STEARIC ACID, ALUMINIUM SALT * OCTADECANOIC ACID, ALUMINIUM SALT		C18.H35.AL1.O2.+2.	310.244

Color	File	Path	Spectrum Type
	EXTRACT_MFP_BSW_K0955.1_768731.0	F:\Mathilde\Extracts_SP	Query Spectrum

Fig. 5. Analysis reports from the comparison of spectra from films generated on the surface of the Anodisc assigned either to polyethylene chlorinated (A) or rubber type 3 (B) against broad commercial databases including polymers, additives and chemicals within the Bruker OPUS software.

2022; Ergas et al., 2023; Geng et al., 2023; Mancuso et al., 2023). To reduce the risk of such the film-alike formation of residual materials in the future, we recommend to avoid the direct concentration of samples after matrix digestion on an analytical filter in any methodological design. Whilst this is optimal for reducing the risk of external contamination, lipid-rich residual materials may still be present and thus interfere with subsequent analysis. Using a series of selected additional filtration or digestion/extraction steps can increase the sample digestion efficiency. For example, Dawson et al. (2020) recently investigated the application of ethanol to improve the solubility of the formed fatty acids and improve the filtration behavior. Our example of the false-positive assignment of lipids and retreatment with oxidation showed that the addition of such simple steps to a method could have a substantial impact on the derived results, and thus their use in any subsequent interpretation for environmental samples.

The formation of lipid films is not only limited to the Antarctic region, KOH digestion or krill and fish samples. The digestion of biological or human tissue samples may form such precipitates in general and impact the selection analysis of potential bio indicator species for monitoring like mussels or fish species (AMAP, 2021; Grøsvik et al., 2022; Kögel et al., 2022; De Witte et al., 2023) in accordance to the novel European Marine Strategy Framework Directive (MSFD) (Galgani et al., 2023) and GESAMP (GESAMP, 2019) guidelines but also for human samples (for example lung tissue (Jenner et al., 2022). The current extend of this issue needs to be addressed in the future.

Due to these low numbers found in the krill samples we could not test the second question, if a decreasing trend in MP pollution can be found towards the Antarctic peninsula. Still, it was found that krill in the EIN region had the highest biomass stomach content but similar MP concentrations as the BSW region with the lowest stomach content. For NWWS we could not find MP particles but a higher stomach content compared to BSW, yet the resulting uncertainties for the EIN and BSW concentrations did not allow any further testing of the data.

As a source for MP, macroplastic has been identified on the shores of islands within the Southern Ocean for decades (Suaria et al., 2020). The first reports about plastic pollution in the Southern Ocean and Antarctica date back to the 1980s (van Franeker and Bell, 1988), but only since 2017 has MP pollution received attention (Absher et al., 2019). MP investigations in the Southern Ocean have demonstrated that MP input from local sources, such as research stations, tourism, fishing and research vessels, is much more significant than from external sources to the north of the Antarctic Circumpolar Current system, which acts as natural barrier (Waller et al., 2017). Supporting this view are dispersal model studies indicating local releases of MP from the Antarctic Peninsula (Lacerda et al., 2019; Kim et al., 2023). Additionally, the predominantly detected MP items in this region consist of polyurethane and polyamide, which are frequently used for surface coatings or insulation panels at research stations or on vessels in the Southern Ocean (Absher et al., 2019; Lacerda et al., 2019). Furthermore, MP fibers from laundry wastewater, released by research stations and ships, have been identified (Waller et al., 2017), and there is an apparent decrease in levels of MP contamination in marine sediments with increasing distance from Antarctic research stations (Munari et al., 2017; Reed et al., 2018). The most prevalent MP material present in these sediments was styrene-butadiene-styrene copolymer, an elastomer that is used widely in waterproofing systems, pneumatic tires, and shoe heels and soles, all of which can be found on research stations. The recorded MP levels in contaminated marine sediments from Antarctic regions with high anthropogenic impact were similar to those in marine sediments from outside Antarctica (Reed et al., 2018), strongly implying the considerable influence of human presence on local MP pollution in the Southern Ocean. Furthermore, in surface water samples collected along the Antarctic Peninsula, a higher MP concentration was detected close to research stations compared to water samples from open ocean areas (Jones-Williams et al., 2020). Still, Cunningham et al. (2022) found several indicators for MP potentially breaching the ACC barrier by analyzing air, subsurface and sediment samples.

The issue of MP in the Southern Ocean has only recently entered scientific and political agendas, and the current state of knowledge is unclear due to a lack of harmonized or standardized protocols for sampling and analyzing (Löder and Gerdts, 2015; Waller et al., 2017; Huang et al., 2023), making it difficult to compare generated data on MP distribution. As a result, the existing data on MP pollution in Antarctica cannot be placed in a broader context, preventing conclusions on the current status of MP contamination in this region. Using the results on MP contamination in the Southern Ocean from studies which provided a detailed quality control of their generated data, the Antarctic marine ecosystem can still be considered as close to a “state zero” (i.e. relatively pristine) environment. Given the global increase in MP in marine environments, it is important to maintain near MP-free ocean regions, if only to serve as a baseline level of MP contamination in our oceans. In this context, it is pivotal to have regional monitoring programs (Lusher and Primpke, 2023) in place, with internationally commonly agreed harmonized/standardized methods for sampling and analyzing MP in organisms, environmental compartments, and areas enabling us to identify any small increases to MP contamination in Antarctica. In this context, the area south of 60°S is governed by the Antarctic Treaty System, with the Convention for the Conservation of Antarctic Marine Living Resources (CCAMLR) being the most important commission for protecting Antarctic marine life. The emerging problem of MP in the Southern Ocean was first introduced to a CCAMLR working group in 2016, resulting in the monitoring of anthropogenic litter at different sites around the Antarctic continent (Waller et al., 2017) and the establishment of an action group by the Science Committee on Antarctic Research (SCAR; Garcia-Garin et al., 2020).

Krill’s large biomass in the Southern Ocean and its feeding basket with a mesh size that allows to filter nano- and microplankton from seawater make it an ideal indicator species to quantify MP in the water column over the long-term. Krill fishing vessels would be an ideal platform to catch krill throughout the year for MP studies, in different regions, which would then be analyzed for MP contamination according to internationally standardized protocols.

However, the biggest problem highlighted for MP research including the Southern Ocean is the current lack of uniform protocols for sampling and subsequent analysis. As showcased in our study this lack of uniformity leads to many shortcomings that make data comparison difficult and ultimately limit the possibilities for conclusions on the extent and dynamics of MP contamination in this region (Cannon et al., 2016; Kelly et al., 2020; Aves et al., 2022). While works on the development of harmonized or standardized protocols and guidelines have been started e.g. in US by SCCWRP (De Frond et al., 2022), in Europe by projects like EUROqCHARM (Aliani et al., 2023) and worldwide by the International Organization for Standardization (ISO, 2020; ISO, 2023), reported first SOPs have mainly be implemented for dedicated spiked tissue sample (e.g. salmon; Thornton Hampton et al., 2023) to simulate the different matrices available for ILCs. Even following these SOPs can yield unexpected implications for the analysis, making it necessary to publish detailed outlines of the methodology used for MP identification in order to make studies comparable and reproducible (Cowger et al., 2020) and to critically assess each method’s strengths and weaknesses. Our study is a prime example of how quickly a highly erroneous quantification of MP could have been produced if not for the application of sufficient quality controls.

5. Conclusion

Our study highlights the impact of residues from a lipid rich diet on the analysis of microplastics by FTIR in krill stomachs. Following generally applied and recommended digestion protocols using KOH and neutralization of the material, we found these may form for the magnified eye invisible film-alike residues on filter surfaces which can be misinterpreted as different synthetic polymers. By quality control and

improved sample and data analysis the initial results of 5–91 MP stomach^{-1} was reduced to 0 to 0.4 MP stomach^{-1}. By scaling the results to typical krill swarm sizes, this would have led to an extreme overestimation in microplastics contamination in the Southern Ocean with unforeseeable wrong conclusions.

Our finding highlights the importance, especially in almost pristine environments such as the Southern Ocean, of a) verifying results very carefully before coming to a final decision on levels of MP contamination, and b) establishing internationally standardized protocols for the sampling and analysis of MP in organisms and habitats of the Southern Ocean.

Both parts are a prerequisite to generate meaningful data to inform policy makers, allowing action to be taken to stop any identified sources of MP input into the Southern Ocean. In order to be able to make reliable statements on the MP loads, it is urgently necessary to develop a monitoring program similar to the Arctic, in which indicator species, as well as regions, are identified to study MP in the long-term and to evaluate MP-present regions versus MP-free areas in the Southern Ocean and worldwide.

CRediT authorship contribution statement

Sebastian Primpke: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation. **Bettina Meyer:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Investigation, Formal analysis, Conceptualization. **Mathilde Falcou-Préfol:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Wyona Schütte:** Writing – review & editing, Methodology, Investigation. **Gunnar Gerdts:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Conceptualization.

Declaration of competing interest

The authors have no competing interest to declare.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Additional experimental materials and methods, including photographs and scheme of experimental setup (Figs. S1 and S2); Spectra of the microplastic particles found in four individual Krill stomachs (Figs. S3-S6); Example of spectrum of a blank sample (Fig. S7). Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.170618.

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