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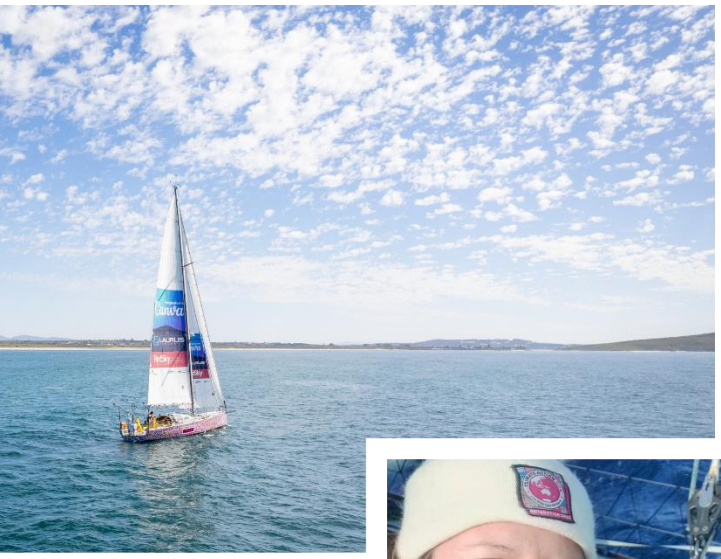
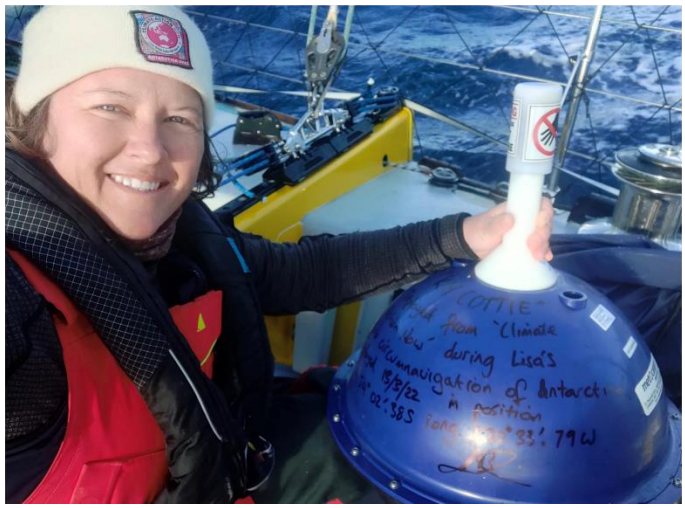
Australian Institute of Marine Science

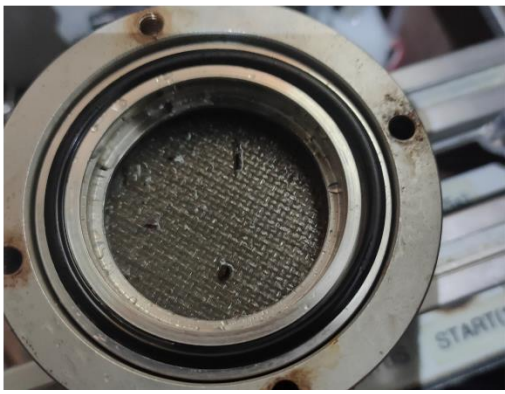
A Validated Method to Quantify Microplastic Contamination in Subsurface Seawater:

A case study sampling the Sydney nearshore under sail

Marina F. M. Santana, Lisa Blair, Samantha Jaworski, Cherie A. Motti







A document prepared for The Clean Ocean Foundation

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Lisa Blair montage; microplastics sampling under sail. Images: L. Blair

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1 SUMMARY OR EXECUTIVE SUMMARY

AIMS, in collaboration with The Clean Ocean Foundation, has completed a proof-of-concept study to facilitate sampling of microplastics in the Southern Ocean (a joint collaborative initiative involving the Australian Institute of Marine Science (AIMS), the Integrated Marine Observing System (IMOS) and solo yachtswoman Lisa Blair). This report presents findings from the opportunistic sampling of subsurface waters along the NSW coastline and provides the first spatial baseline information on microplastic contamination in NSW waters adjacent to estuary outflows and urban outfalls.

Subsurface coastal NSW waters were collected during transit (and under sail) from Brisbane to Albany. Water samples were collected on stainless-steel sample filters of 100 µm aperture size using the SubCtech OceanPack™ RACE® Microplastic Sampler, an emerging technique that allows for the continuous spatial sampling of subsurface seawater within minimal manual intervention. This collaborative pilot project built capability by trialling density flotation and chemical digestion separation methods to process the subsurface samples and retrieve environmental microplastics. Workflow optimisation found repeated chemical digestion employing potassium hydroxide solutions was most effective at microplastic recovery, and validation of this stepwise protocol through spike recovery tests ensured robust data collection.

Microplastics were retrieved from each of the eight samples collected. Polyethylene and polyester fibres were the most prevalent polymer types detected and the highest numbers were recorded adjacent to urban outfalls. This pilot sampling study provides preliminary data on the abundance and type of microplastics in NSW waters, and based on these, the report recommends more detailed spatial and temporal collection in NSW waters to generate a comprehensive baseline of microplastic contamination. It is envisaged such information will critically inform policy and management of land-based plastic run-off and the mitigation of marine plastic pollution in NSW.

Finally, the sampling and processing protocols developed here allow for the extension of the spatial coverage of microplastic data in Australian and Southern Ocean waters.

2 BACKGROUND

The Australian Institute of Marine Science (AIMS) has entered a collaboration with the Integrated Marine Observing System (IMOS) and internationally renowned solo yachtswoman Lisa Blair to collect and quantify microplastics (i.e., plastics <5 mm) in waters important to Australia, including in the Southern Ocean. Microplastics are ubiquitous within the marine environment and have been found in every marine matrix investigated so far, including seawater (Jung et al., 2021; Miller et al., 2017), marine sediment (Fang et al., 2022; Ni'am et al., 2022; Thompson et al., 2004; Van Cauwenberghe et al., 2015), sea ice (Kanhai et al., 2020; Peeken et al., 2018; Zhang et al., 2022b), and a multitude of organisms at every trophic level (Miller et al., 2020), including those for human consumption (Dawson et al., 2021).

With marine plastic contamination predicted to increase with projected increases in plastic production and use, scientists, managers, and the broader public are increasingly interested in understanding the status and temporal trends of plastic contamination in the marine environment. However, there is a lack of comprehensive spatial and temporal characterization. Lisa Blair's circumnavigation of Antarctica provided a rare and unique opportunity to capture a continuous subsurface seawater sample set to quantify microplastic contamination in the Southern Ocean, facilitated by the specially designed *in situ* SubCtech OceanPack™ RACE® Microplastic Sampler. While previous projects have sampled Antarctic waters (Suaria et al., 2020) and microplastics have been captured using said equipment (Lenz, 2020; Tanhua et al., 2020), no study has applied a continuous (24 hours, 7 days a week) sampling regime for microplastic assessments. As part of the IMOS New Technology Proving Microplastics Project (NTPMP), established to operationalise monitoring of marine microplastic contamination in Australian waters, AIMS has developed and refined workflows, standard operating procedures and methods to analyse surface seawater tow samples (Kroon et al., 2018a; Miller et al., 2022a). The design and mode of operation of the SubCtech OceanPack™ RACE® Microplastic Sampler is markedly different from tows commonly used by AIMS and the NTPMP, and modification of existing methods or development of new ones for subsurface seawater sample processing was required. The aim of this project was to adapt AIMS' existing methodologies, including those developed for NTPMP, and validate them for the processing of samples collected by the *in situ* Microplastic Sampler. To facilitate this, a second collaboration was established between AIMS and The Clean Ocean Foundation. Subsurface waters were opportunistically sampled off the coast of Sydney (New South Wales; NSW) as Lisa Blair sailed from Brisbane (Queensland) to Albany (Western Australia), the location for her departure for Antarctica. The pilot study tested the hypothesis that nearshore waters adjacent to estuary outflows and urban outfalls are a hotspot for microplastic contamination.

The intent of this study was to establish a validated method for continuous sampling and processing of subsurface seawaters to quantify microplastic contamination in different marine environments. As a case study, the project generated baseline information on microplastic contamination in NSW coastal waters and provides insight into the relationship with land-based plastic sources.

3 METHODS

3.1 Participants

Party	Personnel
Lisa Blair Sails the World	Lisa Blair
Ocean Race	Stefan Raimund
Clean Ocean Foundation	John Gemmill
IMOS	Michelle Heupel
AIMS	Frederieke Kroon (retired Principal Investigator) Cherie Motti (Principal Investigator) Marina Santana (Technical Lead) Samantha Jaworski (Technician) Keegan Vickers (Technician)

3.2 Equipment and materials

A microplastics sampling instrument, similar to that installed on two yachts that competed in The Ocean Race (Tanhua et al., 2020), was used here. The SubCtech OceanPack™ RACE® Microplastic Sampler (SubCtech; SubCtech) (Figure 1) is designed for fast throughput, semiautomatic, autonomous monitoring and can be installed on any type of vessel. In December 2021 the unit was installed onboard the yacht "Climate Action". The technique involves pumping subsurface water over a coarse stainless-steel filter before flowing over a cassette consisting of a custom-built stainless-steel sample filter (SSSF) with an opening diameter of 44 mm and aperture mesh-size of 100 µm (bbe Moldaenke GmbH). Data, including flow rate, Global Positioning System (GPS) coordinates and time stamp, were recorded electronically.

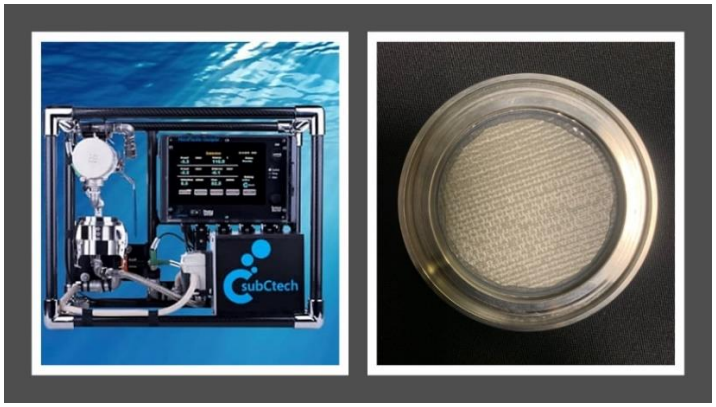

Figure 1 SubCtech OceanPack™ RACE® Microplastic Sampler and SSSF

Pre-used (i.e., dirty, encrusted residue present) SSSF (as supplied by bbe Moldaenke GmbH) plus silicon O-rings were cleaned as follows. First, the black O-ring was removed from the SSSF and rinsed with minimal acetone to remove residual oils. Cleaned O-rings were air dried in a covered glass petri dish, inspected by microscopy (LEICA M80, 3.2 to 4x magnification) to assess cleanliness, and

thereafter wrapped in aluminium foil to protect from extraneous laboratory contamination until needed. Second, the SSSFs were placed into a furnace (Ceramic Engineering) at 450°C overnight. The high temperature resulted in the incineration of the encrusting residue and particulate matter, as well as the silicone sealant used to secure the stainless steel filter mesh (comprising of three mesh of welded together, the top two >1 mm aperture and the third 100 μm; hereafter referred to as the welded mesh) into the outer stainless steel casing. Once cooled (~2-4 h), the SSSF were air-blasted to remove ash and dismantled by removing the welded meshes from the outer casing (Figure 2A and B). Each component was immersed in a bath of 10 M sodium hydroxide (NaOH, Sigma-Aldrich, RG, CAS 1310-73-2) at 98°C for 30 mins. Next, welded mesh and casings were carefully transferred into a bath of 0.001 μm filtered (reverse osmosis, RO) water, rinsed twice with RO water, and transferred to a 10% hydrochloric acid (HCl, Sigma-Aldrich, RG, CAS 7647-01-0) bath for 3 mins (Figure 2C). Again, each component was rinsed five times with RO water, covered with aluminium foil and dried in an oven (Thermo Scientific, Heratherm OGH180) overnight at 100°C. In a laminar flow cabinet (Thermo Scientific, HERAGuard Eco), cooled welded mesh were examined by microscopy (LEICA M80, 3.2 to 4x magnification) to confirm they were free of debris. Clean welded meshes were reassembled by positioning them on the seat of the casing, and were secured in place with silicon (Selleys Ezi Press 100g Wet Area Silicone Sealant). For quality assurance and quality control (QA/QC), all cleaned reassembled SSSF were inspected by microscopy. Finally, SSSFs were bagged individually in clean aluminium Mylar Ziploc bags (80x100 mm; Vivo Packaging) from which they were only removed for sampling. Mylar bags were placed in a Pelican case and secured in place to minimise movement during transit.

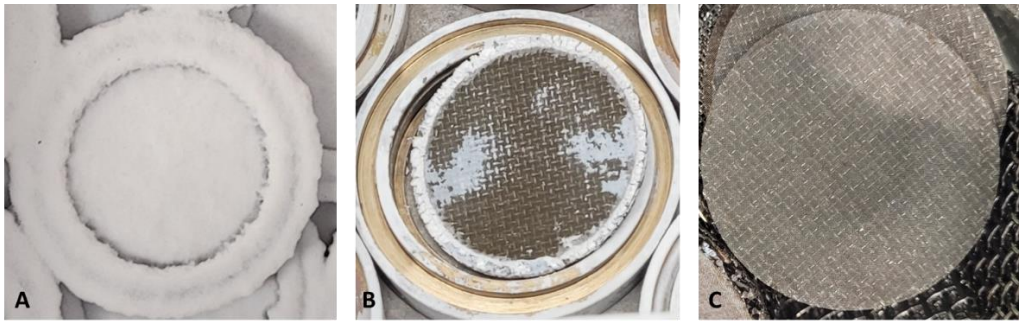

Figure 2 Stainless steel sample filters during the cleaning process. A) Residual ash left from incineration, B) welded mesh removed from the outer casing and C) welded mesh after cleaning.

3.3 Sample Collection

The Sydney coastline has three main estuary outflows and wastewater treatment plant outfalls (North Head, Bondi, and Malabar; Figure 3) with low quality discharge (primary treatment only). Eight hour-long near-continuous seawater sampling events were conducted following a north to south transect (working area between -33 35.701 151 26.5319 and -34 18.9045 151 09.9813; Figure 3) running adjacent to these discharge outfalls, each transect representing locations likely to be contaminated with land-based materials. Detailed latitude and longitude coordinates of the start and end of each sampling transect are provided in Table 1. Oceanographic parameters such as temperature, salinity, conductivity, CO_2 and chlorophyll content were simultaneously collected (details of these are not reported here but have been curated and are available on request).

Table 1 Starting and finishing points (latitude and longitude) for the eight sampling transects

Transect number	Transect start coordinates		Transect end coordinates	
	Latitude	Longitude	Latitude	Longitude
T_1	-33 35.7	151 26.5319	-33 41.77	151 24.2069
T_2	-33 45.01	151 23.6283	-33 50.3	151 22.716
T_3	-33 50.66	151 22.6577	-33 55.43	151 21.1784
T_4	-33 55.72	151 21.0989	-34 00.93	151 21.9153
T_5	-34 01.32	151 22.0003	-34 05.98	151 22.6127
T_6	-34 06.27	151 22.3659	-34 10.43	151 18.3489
T_7	-34 10.77	151 18.0643	-34 14.82	151 14.3873
T_8	-34 15.12	151 14.0555	-34 18.9	151 09.9813

Continuous microplastics sampling was achieved by filtration of pumped seawater over a 100 μm SSSF (Figure 1). Filters were manually exchanged every hour (as quick as possible, ~3 min, to minimize exposure time to the environment). For QA/QC, and to correct for any extraneous airborne contamination from the yacht, one SSSF was used as control. The control SSSF was removed from its sealed Mylar Ziploc bag and placed next to the sampler each time a SSSF was exchanged i.e., the eight SSSF were collected with the same control SSSF exposed each time (i.e., ~3 min x 9 SSSF changes). After installation of the next SSSF, the control SSSF was returned to its original labelled Mylar Ziploc bag and resealed. No plastic gloves were used during the process as per Tanhua et al. (2020). Sample collection did not require any permits as locations are outside the adjacent Marine parks (Port Stephens just north of Newcastle or Jervis Bay south of Wollongong) or Aquatic reserves.

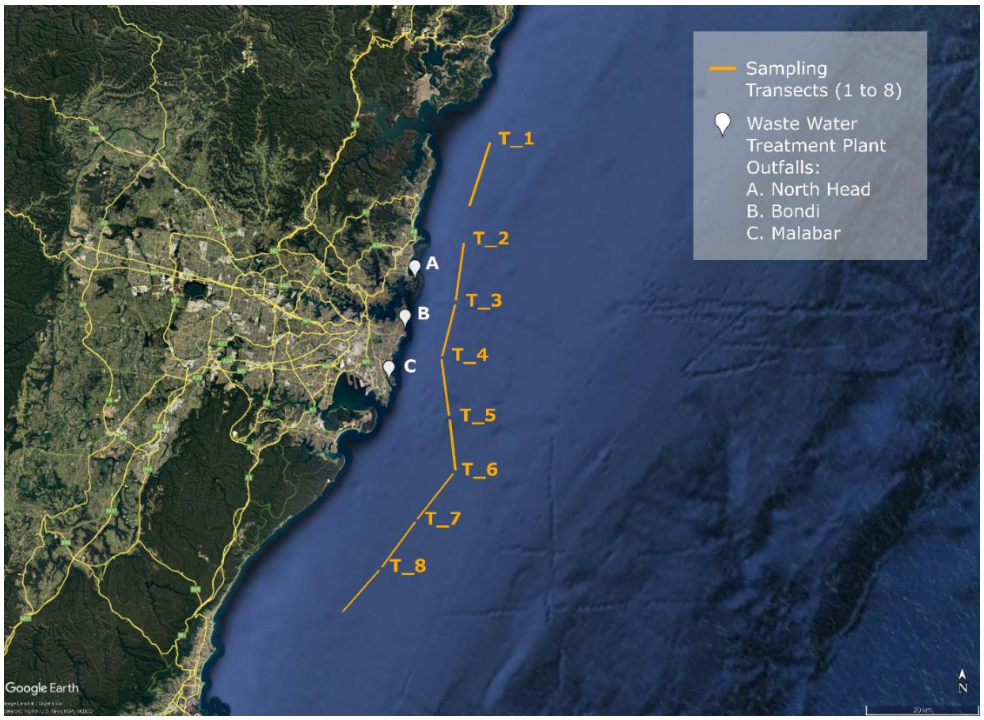

Figure 3 NSW sampling transects and location of the three main wastewater outfalls of Sydney.

3.4 Sample processing and method validation

3.4.1 Sample storage and pre-preparation at AIMS

Stainless steel sample filters (SSSF) received by AIMS were kept in their sealed Mylar Ziploc bags at 4°C until processing. Prior to laboratory processing, each SSSF (including the control SSSF) was removed from its bag in a laminar flow cabinet, placed in a pre-cleaned glass petri dish and lightly sprayed with absolute ethanol (UNIVAR, AR, CAS 64-17-5; filtered over 0.45 µm PTFE MERCK membrane) to prevent mould growth. Wetted SSSF were left to dry in a sealed glass desiccator overnight. Initial visual inspection established (biological) debris was present on the SSSFs relative to the SSSF control.

3.4.2 Sample processing and method validation

Two methods routinely used by AIMS to process dry or high-load environmental samples and proven to not impact the physical and chemical integrity of microplastics were assessed to establish which was most suited to the processing of subsurface seawater samples collected on SSSF. These methods are density flotation and alkaline digestion (Lusher et al., 2020; Santana et al., 2022; Stock et al., 2019). For density flotation, SSSF were immersed in 1.7 $g.cm^{-3}$ potassium iodide (KI, Sigma-Aldrich, AR, CAS 7681-11-0) brine solution. This method expedites floating of plastics. For alkaline digestion, SSSF were immersed in 10% potassium hydroxide (KOH, Sigma-Aldrich, AR, CAS 1310-58-3) solution to loosen and digest any organic material that may potentially trap putative microplastics on the SSSF and impede their recovery.

A high debris loading was pre-empted, based on personal communications with The Ocean Race. Therefore, the two methods were applied in a stepwise matrix (Lusher et al., 2020) (Table 2) to improve their performance. The following processing workflow was applied: the SSSF was immersed in ~200 mL of filtered (0.45 µm) reagent overnight at 40°C, followed by 2 h of sonication. The SSSF was then removed and the solution filtered through a custom-designed tiered stainless-steel filtration apparatus fitted with 250, 77 and 26 µm stainless-steel mesh (25 mm diameter) as per Schlawinsky et al. (2022). The SSSF was immersed in a second volume of reagent and again treated for 2 h at 40°C, followed by 2 h of sonication and filtration through a second set of size tiered (250, 77 and 26 µm) stainless-steel mesh (Table 2).

To validate the four stepwise protocols, a spike-recovery test was first conducted to establish recovery rates of test microplastics from dummy SSSFs (in addition to the eight samples and one control). Microplastics commonly found in the marine environment (Rochman et al., 2019) were used for this test, including blue polyester (PEST, 0.5 to 1 mm) and red polypropylene-vistalon copolymer (PP-vistalon, 1 to 1.5 mm) fibres, and grey polyvinylchloride (PVC, 1 to 2.5 mm) and blue PP fragments (0.5 to 1 mm). Three replicate dummy SSSFs per protocol were prepared by filtering 1 L of 0.45 µm filtered seawater spiked with five microplastics of each microplastic type (n = 25 per dummy SSSF). Triplicate spiked dummy SSSFs were then progressed through the processing workflow as described in Table 2. All three stainless-steel mesh collected for each dummy SSSF (n = 3 per replicate, n = 9 per protocol)

were visually assessed and microplastics identified by stereomicroscopy (Leica MZ16A, Leica DFC 500, Leica Application Suite LAS 4.4.0) and counted to calculate microplastic recovery rates. Recovery was considered adequate if ≥70% of microplastics were retrieved (Santana et al., 2022).

Table 2 Processing methods and protocols assessed for the separation of microplastics

Protocol	Step	Method	Reagent	Immersion time (h)	Immersion temperature (°C)	Sonication time (h) and temperature (°C)	Tiered filtration µm	Transect number
1	1	density flotation	KI	>12	40	2, 27	250, 77, 26	T_1
	2	density flotation	KI	2				T_2
2	1	density flotation	KI	>12				T_3
	2	alkaline digestion	KOH	2				T_4
3	1	alkaline digestion	KOH	>12				T_5
	2	alkaline digestion	KOH	2				T_6
4	1	alkaline digestion	KOH	>12				T_7
	2	density flotation	KI	2				T_8

The validated protocols were used to process the eight samples. The eight SSSF were divided into four sample sets, the first set (T_1 and 2) to be processed by repeated density flotation (protocol 1), the second (T_3 and 4) by density flotation followed by alkaline digestion (protocol 2), the third (T_5 and 6) by repeated alkaline digestion (protocol 3) and the fourth (T_7 and 8) by alkaline digestion followed by density flotation (protocol 4) (Table 2). Tiered mesh from all eight samples (n = 6 per sample, n = 48 total) were visually inspected and assessed by stereomicroscopy, and putative microplastics counted and chemically characterized as described below (Section 3.5). Items confirmed to be microplastics were then sized from photos by ImageJ.

The influence of each stepwise protocol on processing efficiency was visually assessed for each of the eight SSSF. Efficiency was determined based on the amount of material liberated from the SSSF (assessment according to the ranking system described in Santana et al. (2022)). This was done by visually estimating the residual debris on the SSSF (i.e., how clean it is after processing) and the debris loading on each of the tiered 250, 77 and 26 µm mesh. Logistical aspects of each protocol were also considered as part of the assessment, specifically processing times, including the time required to visually and chemical identify retrieved microplastics, and costs.

3.5 Microplastics assignment, characterization, and levels of contamination in samples

Items retrieved during sample processing were visually analysed with stereomicroscopy (Leica MZ16A, Leica DFC 500, Leica Application Suite LAS 4.4.0) to identify, record, and characterize (by shape, size and colour) putative microplastics. Items were considered putative microplastics based on their physical features (Noren, 2007; Santana et al., 2022). Thereafter, putative microplastics were individually analysed by Attenuated Total Reflectance Fourier transform infrared spectroscopy (ATR-FTIR, PerkinElmer Spectrum 100) and chemically assigned using a commercially curated library for

synthetic polymers, additives, and textile products (NICODOM). Following the workflow proposed by Kroon et al. (2018b), items were confirmed as microplastics if < 5 mm in size and having a spectral match to a plastic polymer. Confirmed microplastics were then assessed against QA/QC measures (see below) to exclude potential laboratory-derived contamination and determine final counts of microplastic contamination per SSSF.

3.6 Quality Assurance and Control (QA/QC)

3.6.1 Contamination prevention and control

During sample collection and processing, potential contamination of environmental samples with microplastics associated with field vessels and laboratories (including in the air) is possible and need to be controlled to ensure data robustness and accurate estimates (Prata et al., 2020). Hence, a series of measures were adopted in this study to prevent, control and exclude extraneous microplastic contamination from final results. Following Kroon et al. (2018b) and Santana et al. (2021), the use of plastic items during sample collection and processing was avoided where possible. Filters and tools made of stainless steel and glass were used in preference. Similarly, clothing, including laboratory coats, were preferably made of natural-based fabric such as cotton. When plastic use was inevitable, the item was photographed, and a sample taken and physically and chemically characterized. All data describing potential contaminant sources were included in a project-specific contaminant library used to control for extraneous microplastics found in samples. In the field, a blank SSSF was exposed to the air every time a SSSF was exchanged in the Microplastic Sampler. This field control SSSF was processed in the laboratory by rinsing with Milli-Q water into the stainless-steel filtration apparatus fitted with a 26 μm stainless-steel mesh and analysed for microplastics. Any contaminating microplastics were excluded from samples following the established protocol (Kroon et al., 2018b). The field control SSSF was deemed to only need rinsing with Milli-Q since this was only exposed to the air and not placed under pressure as would be experienced by those installed in the microplastic sampler. In the laboratory, tools and benches used for sample processing were cleaned following AIMS standardized procedures. Processing controls (glass petri dishes containing Milli-Q water) were placed adjacent to the work area to capture any extraneous airborne microplastic contaminants. At all times when SSSF were exposed to air, these Milli-Q laboratory controls were also exposed; a new control was used each day of processing. Sample handling was always conducted in a laminar flow cabinet to further minimise contamination from airborne sources. Airborne microplastics recovered from field and laboratory SSSF controls and laboratory blanks were also included in the contaminant library and thereby excluded from the final reported data during analysis.

4 RESULTS AND DISCUSSION

4.1 Development of a protocol to separate microplastics from subsurface seawater samples collected using the Microplastic Sampler.

There are a myriad of reports detailing the development and validation of methods to separate microplastics from environmental samples (Hooriabad Saboor et al., 2022; Miller et al., 2017; Nabi et al., 2022), with the sample matrix ultimately dictating which is the most appropriate (Dawson et al.,

2020; Dehaut et al., 2016; Miller et al., 2021; Ruggero et al., 2020; Santana et al., 2022). For some environments microplastic monitoring methods have been applied to measure the abundance and composition of microplastic contamination, both temporally and spatially, and record any changes concurrent with policy implementation (Averbuj et al., 2015; Herath et al., 2022; Miller et al., 2022a). IMOS and AIMS are currently developing and operationalising a microplastics monitoring program to assess microplastic contamination in Australian marine waters (Figure 4), and in collaboration with Lisa Blair and the Clean Ocean Foundation, have extended this to include a pilot study of NSW waters.

The separation of microplastics from environmental water samples is contingent on the method collection (Kroon et al., 2018a; Whitaker et al., 2019), with estimates of contamination levels dependent on the local (i.e., site-specific) physicochemical properties (Miller et al., 2022a). Current methods employed by the AIMS microplastics group to separate microplastics from marine waters are tailored to samples collected by neuston net tows (i.e., 10-15 min transects, air-water surface interface, horizontal tow dependent on speed (<4 knots) and currents, net mesh aperture 355 μm) (Kroon et al., 2018a; Miller et al., 2022a). Collection using the *in situ* SubCtech OceanPack™ RACE® Microplastic Sampler is inherently different (i.e., one hour or more transects, subsurface, pumped at approximately 1 L min^{-1}, SSSF aperture 100 μm) and requires adaptation of the processing methods.

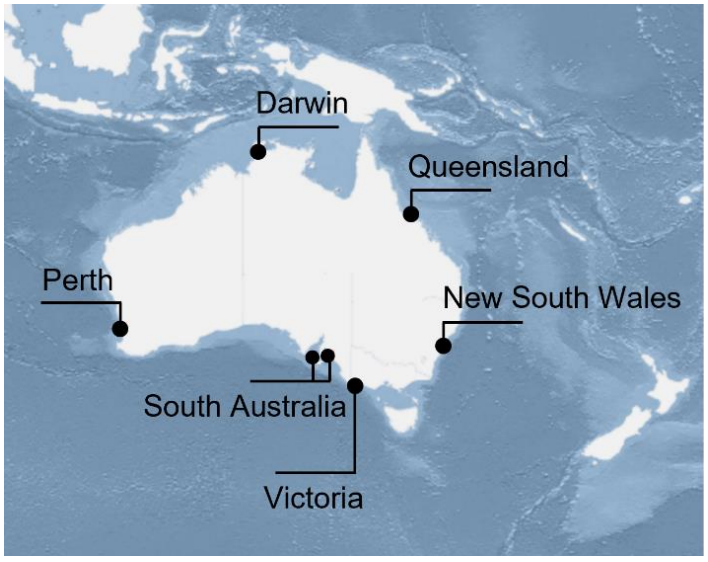

Figure 4 Sampling sites from IMOS and AIMS microplastics monitoring in Australian marine waters. Map available on the AODN portal (aodn.org.au), where curated microplastics data is publicly available.

A priori assumptions, based on the literature ((Frias et al., 2020; Gago et al., 2019; Gulizia et al., 2022; Miller et al., 2022a; Miller et al., 2017; Santana et al., 2022)), identified KI density flotation and alkaline KOH digestion as the two most promising methods to trial. A further assumption was made regarding the layered welded mesh used in the SSSFs, that is, the loading on each overlapping set of meshes is expected to be more difficult to retrieve if items are trapped between the two mesh. Therefore, the two chosen methods were trialled in a stepwise matrix (Lusher et al., 2020) (Table 3) allowing greater opportunity for recovery. Recovery rates for each of the four possible stepwise protocols were determined with spiked recovery samples and validity proven. The KOH → KOH protocol returned the overall highest recovery rates. However, this was not deemed to be different from the other three protocols, which also performed well, all recovering > 70% of spiked microplastics (as per criteria from Santana et al. (2022). For all protocols, PEST fibres returned the lowest recovery rates. This is a

common trait in textile fibres, and can be explained by the narrow width which might facilitate fibres passing through the 26 µm filters if positioned vertically during filtration (Schlawinsky et al., 2022). All other test microplastic types, regardless of their shape and polymer properties (i.e., density) had recovery rates above 90%. Fragments of PP were 100% recovered by all methods tested. Based on these results, repeated stepwise processing through two rounds of microplastic separation proved to be effective and a third step was considered unnecessary.

Table 3 Results from spike-recovery tests conducted for the four stepwise protocols tested (n = 3 replicate tests per protocol, five microplastics per test microplastic type and replicate [n = 20]). Recovery rates reported as average percent (± standard deviation) of recovered microplastics per type of microplastic spiked and method tested. KI = potassium iodide, KOH = potassium hydroxide, PEST = polyester, PP = polypropylene, PVC = polyvinylchloride.

Spiked Microplastic	KI → KI	KI → KOH	KOH → KOH	KOH → KI
PEST	80.00 ± 16.33	73.33 ± 9.43	86.67 ± 9.43	80.00 ± 16.33
PP-vistalon copolymer	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	93.33 ± 9.43
PVC	93.33 ± 9.43	100.00 ± 0.00	100.00 ± 0.00	93.33 ± 9.43
PP	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Following the successful recovery of spiked microplastics, all four protocols were used to process the SSSF (as per Table 2). Qualitatively, post-processing, all SSSF were visually deemed to be clear of debris. A similar number of microplastics were found on all SSSF processed with KI → KOH, KOH → KI and KI → KI (samples 1-2 = 21.5±2.5; samples 3-4 = 15±7, and samples 7-8 = 12.5±1.5). In comparison, SSSF 5 and 6, processed using the KOH → KOH protocol, returned a higher average yield, with 51.5 ± 27.5 microplastics per sample (Figure 5).

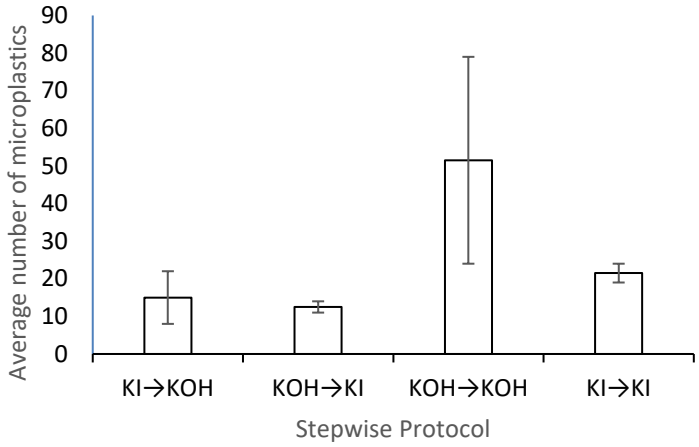

Figure 5 Number of microplastics (average ± standard deviation) retrieved per processing protocol. Values correspond to final microplastic counts, with extraneous contamination from the field collection and laboratory analysis excluded.

Overall, for each SSSF, the average number of microplastics retrieved from each step of the workflow was similar (Figure 6), showing the second step to be as important as the first. This pattern was observed regardless of whether the microplastic load was low (e.g., sample filter 2: step 1 = 6 vs step 2 = 2) or high (e.g., sample filter 5: step 1 = 42 microplastics vs step 2 = 37 microplastics). Microscopy inspection of the tiered stainless-steel meshes revealed few items were trapped on the 250 µm mesh, with the majority being trapped on the 77 µm mesh, followed by the 26 µm mesh filters (Figure 7).

Microscopy inspection of the SSSFs after the second processing step found no evidence of any retained microplastics.

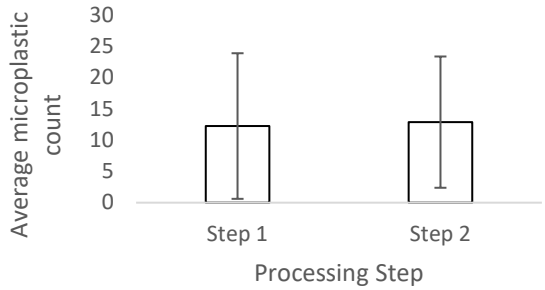

Figure 6 Average number of microplastics (± standard deviation) retrieved per processing step. Values correspond to final microplastic counts, with extraneous contamination from the field collection and laboratory analysis excluded.

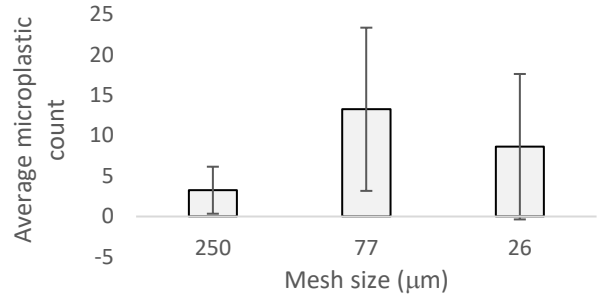

Figure 7 Average number of microplastics (± standard deviation) retained on the tiered stainless-steel mesh. Values correspond to final microplastic counts, with extraneous contamination from the field collection and laboratory analysis excluded.

Initial assessment of the protocols suggested KOH → KOH (used to process SSSF 5 and 6) to be the preferred stepwise protocol to process subsurface seawater samples collected with the *in situ* Microplastic Sampler. However, SSSF 5 was found to skew the data, it having a substantially higher number of microplastics (79 microplastics from T_5) than SSSF 6 collected in the subsequent transect (24 microplastics from T_6; Figure 8) or SSSF 4 collected in the previous transect (14 microplastics from T_4; processed using KI → KOH). Microplastic loadings on the other five SSSF were similar to SSSF 4 and 6, ranging between 8 to 24 microplastics. This suggests there is minimal difference in the efficiency of the four protocols to liberate microplastic debris from SSSF, and that the higher count for SSSF 5 is potentially a result of a higher microplastic contamination at the spatial location from which it was collected.

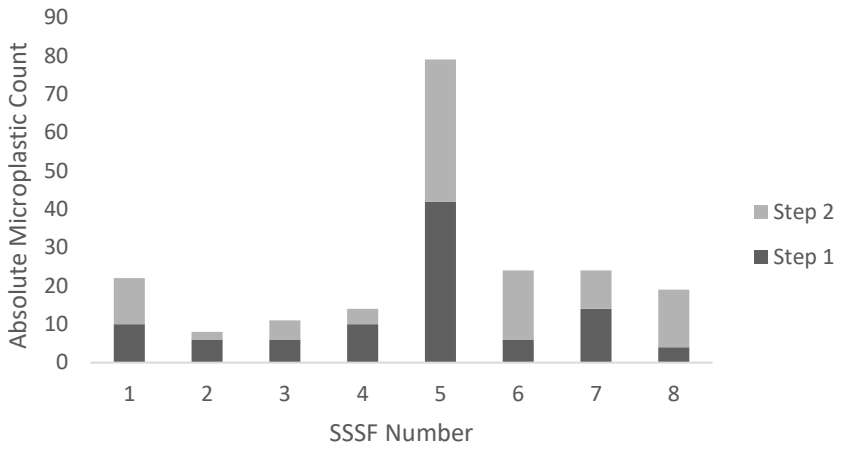

Figure 8 Number of microplastics (absolute numbers) retrieved per stainless steel sample filter (SSSF 1 to 8) per method. Values correspond to final microplastic counts, with extraneous contamination from the field collection and laboratory analysis excluded. SSSF 1 and 2 were treated stepwise with KI→KOH, SSSF 3 and 4 with KOH→KI, SSSF 5 and 6 with KOH→KOH, and SSSF 7 and 8 with KI→KI. KI = potassium iodide, KOH = potassium hydroxide.

The four stepwise protocols tested here (each having two processing steps and filtered over size-tiered mesh) were all deemed suitable for the liberation and retrieval of microplastics from subsurface seawater samples collected on SSSF using the SubCtech OceanPack™ RACE® Microplastic Sampler. All performed well under the same processing timeframe and using the same laboratory techniques and equipment. The processing time, from initial immersion through to chemical analysis, was similar for SSSF 1-4 and 6-8, taking up to five days per SSSF. The higher debris loading on SSSF 5 required an additional 2 days to complete the microscopy and FTIR; in this instance the variation in processing was a function of the high debris loading and was not influenced by the protocol applied. Consideration was given to each reagent's properties: KOH is a caustic substance, but can be safely handled at 10%, KI is hazardous if ingested. Both reagents can be safely handled following standard laboratory practices. Cost wise, the KOH → KOH protocol is economically more efficient. With processing time, efficiency and chemical safety being approximately equal, the KOH → KOH protocol proved to be the method of choice based on its economic value. The KOH → KOH protocol is recommended to process SSSF collected with the SubCtech OceanPack™ RACE® Microplastic Sampler. A Standard Operating Procedure (SOP) specific to processing subsurface seawater samples that will be collected by Lisa Blair at the Southern has been developed and is available for consultation upon request, along with the final NSW dataset (available on request).

4.2 Microplastic presence, levels of contamination and characteristics in NSW waters: are waters adjacent to urban outfalls a hotspot for microplastic contamination?

4.2.1 Microplastic abundance in NSW waters

On average, 66.83 ± 1.12 L (± standard deviation) of seawater was sampled per one hour transect. In total, 201 microplastics were retrieved from the eight SSSFs (Figure 9), with an average concentration of 381.80 ± 335.58 microplastics m^{-3} per sampling transect. Concentrations varied from 118.45 to 1177.91 microplastics m^{-3}, with transects T_2 and T_5 having the lowest and highest levels of

microplastics, respectively. Based on the existing data from subsurface seawaters (Desforges et al., 2014; Tanhua et al., 2020; Zhang et al., 2022a), microplastic concentrations found in this pilot study are considered high and are possibly related to the proximity of the sampling to Sydney, a highly urbanized area with associated estuary outflows and wastewater treatment outfalls. In a study conducted in the northeastern Pacific Ocean and coastal British Columbia, concentrations of microplastics in subsurface seawater varied from 8 microplastics m^{-3} in offshore regions to 9200 microplastics m^{-3} in coastal areas close to urbanization (Desforges et al., 2014). In remote Antarctica microplastic concentrations in subsurface seawaters were reported to be on average 1.66 ± 1.20 microplastics m^{-3} (Zhang et al., 2022a). Nevertheless, other environmental factors, other than or in addition to proximity to source (e.g., urbanization), can also influence microplastics abundance and distribution. In a similar study using the same SubCtech OceanPack™ RACE® Microplastic Sampler, Tanhua et al. (2020) found levels of microplastic contamination across the world varied from 0 to 349 microplastics m^{-3}, and postulated the highest concentrations were likely associated with major ocean currents and not proximity to coastlines and urbanized areas. It is important to note that, in comparison to other compartments such as the surface seawater, only a few studies have collected and analysed microplastics from subsurface seawaters. This precludes comprehensive comparisons and a diligent understanding of microplastic distribution patterns and influences of sources in this abiotic compartment, highlighting the need for more studies.

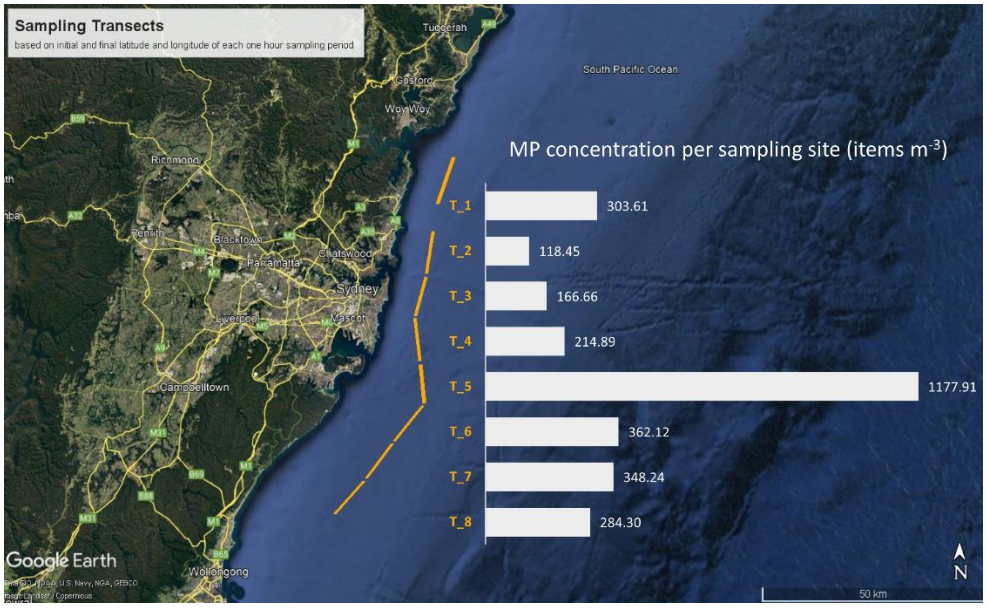

Figure 9 Average microplastics concentration across NSW transects T_1 to 8 (units are microplastics per cubic metre per sampling transect).

Comparison of microplastic numbers across the eight sampling transects found contamination levels to be similar, although there was fluctuation observed, with a spike in T_5. An initial drop was observed from transect T_1 to 2, with a linear increase over transects T_2, 3 and 4. An exponential increase in the number of microplastics found was observed in T_5, which then declined, returning back to the averaged baseline level in transects T_6, 7, and 8 (Figure 9). The spike in contamination in T_5 could be related to its southern position in relation to the wastewater outfalls and estuarine outflows (Figure 3). Local oceanographic parameters and processes effect both estuaries and wastewater effluent plumes, which are usually transported downstream while also dispersed by currents e.g., the East Australian Current (Wijeratne et al., 2018), waves, oceanic turbulence, and

mixing processes (Middleton et al., 1997). Similarly, these oceanographic conditions could be influencing the distribution of the microplastics in the region, increasing their levels from T_1 to 5 before further dispersal. Given these oceanographic conditions can change over time (Middleton et al., 1997), the concentrations and spatial distribution observed here may represent a point-in-time baseline for the region and more sampling events are required to establish temporal patterns of microplastic contamination. With additional data points (i.e., more frequent long-term sampling of north to south and west to east transects), hydrodynamic modelling could also be used to confirm the relationship between plumes and microplastics transport, and further guide mitigation strategies.

Overall, the high load of microplastics in all eight transects, as compared to other subsurface seawater samples globally, corroborates the positive relationship previously reported between highly urbanized coastal areas and high marine microplastic contamination (Desforges et al., 2014; Li et al., 2022). Despite the knowledge that riverine outflows can act as source of microplastics for Australian marine waters (Miller et al., 2022b), as this pilot study did not conduct a comprehensive sampling regime it is not possible to confirm the presence of microplastics hotspots in NSW marine waters adjacent to river outflows and wastewater outfalls. The spatial distribution of microplastics observed in the nearshore waters of the Sydney region suggests these contaminants are possibly being supplemented from plumes associated with the local estuary outflows and/or wastewater outfalls, and being transported, likely in a southerly direction, with dispersal and dilution occurring within distances of 20-40 m. Monitoring of the plumes, including sampling across the plume gradient, is needed to establish if they are indeed the source of microplastics and at what scale dispersal and dilution is occurring.

4.2.2 Potential risks associated with microplastics found in NSW waters

Subsurface seawater samples contained secondary microplastics (i.e., originating from the breakdown of larger plastic debris); no primary microplastics (i.e., manufactured beads) were detected (Figure 10). Similar numbers of fragments (48%) and fibres (52%) were found, with five of the eight sampling transects having more fibres than fragments (Figure 11A). Sizes of these varied from 55.5 µm to 12.4 mm, with 68% of items measuring less than 1 mm (68%), 20% between 1 and 2 mm, and 12% considered to be macroplastics (< 2 mm) (Figure 11B). The most abundant microplastic colours were transparent (37%), blue (13%), white and green (11% each) and black (7%) (Figure 11C). Together, other microplastic colours including red, yellow, orange, and multicoloured items such as blue and white microplastics represented the remaining 21% of the total. Thirty-eight different microplastics polymer types were found. Of the total 201 items, 143 were synthetic (i.e., a plastic polymer) and 56 semi-synthetic (i.e., a composite of plastic polymer and natural polymer, mostly cellulose). The most abundant polymer type found was polyethylene glycol (19%), followed by polyester (10%), polypropylene (9%), polyethylene (8%), rayon (7%), rayon-nylon copolymer and polysiloxane (6% each) (Figure 11D). Other polymer types, such as epoxy, nylon, polyurethane, and cellulose synthetic polymer composites consisting of nylon and polyester made up a combined 35% of the total microplastics. The physical and chemical characteristics of the microplastics found in these samples are aligned to what is currently reported on for marine environments worldwide, including Australian waters. For example, fragments and fibres have been reported as being equally distributed in the mid-column at Lizard Island, located in the Northern region of the Great Barrier Reef (GBR) (Santana et al., 2021). High abundance of microplastics smaller than 1 mm are often reported across different ecosystems worldwide (Avio et al., 2015; Corami et al., 2021; Ding et al., 2019; Garnier et al., 2019; Su

et al., 2020). Polyethylene and polyester are also commonly reported as major marine microplastic contaminants (Exposito et al., 2021; Kroon et al., 2018b; Reisser et al., 2013; Zhang et al., 2022a), including in long-term monitoring assessments of Australian waters (Miller et al., 2022a). Polyester fibres specifically are commonly related with textile sources, which are often suggested to be introduced into marine environments via wastewater effluents (Browne et al., 2011).

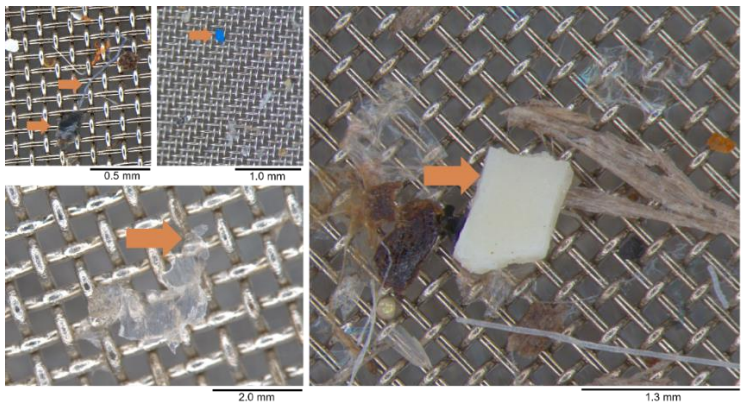

Figure 10 Examples of microplastic fibres and fragments retrieved from subsurface coastal waters of NSW, collected using the SubCtech OceanPack™ RACE® Microplastic Sampler. Orange arrows indicate microplastics that were visually identified and confirmed using Fourier transform infrared spectroscopy.

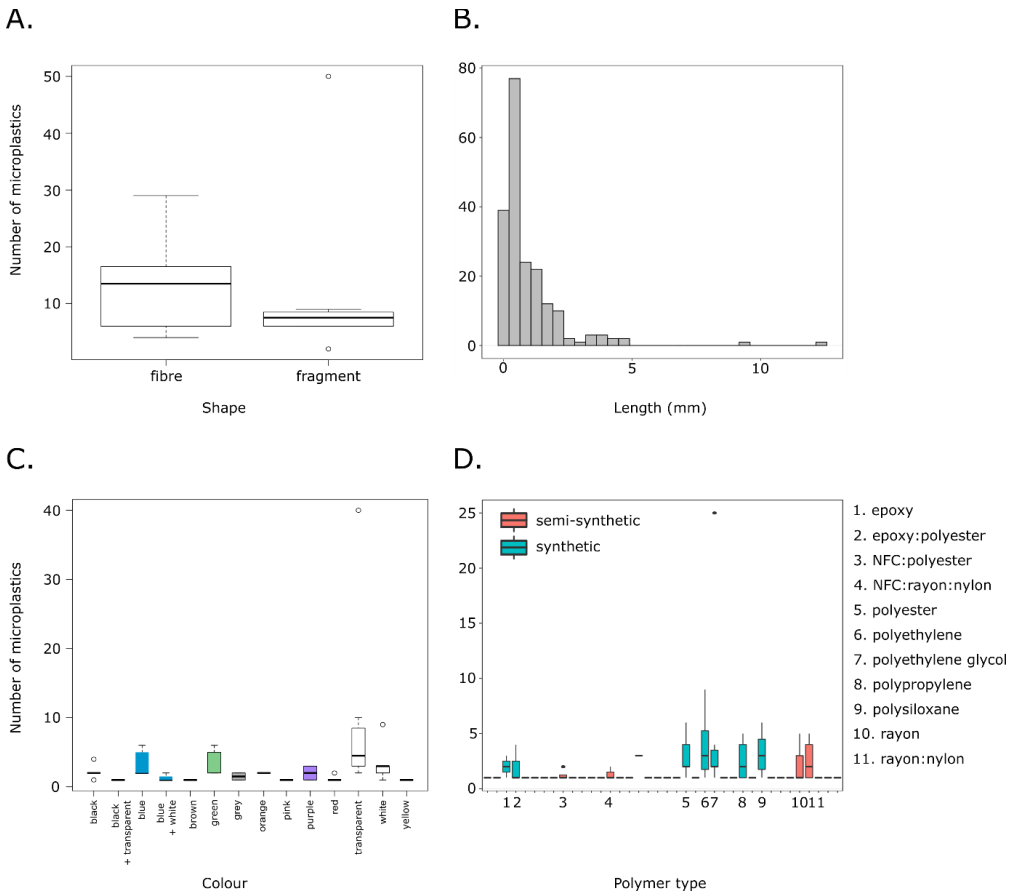

Figure 11 Number of microplastics retrieved according to (A) shape, (B) length (mm), graphed as a histogram given the continuous nature of the sizing data, (C) colour, and (D) polymer type. Boxplots in A, C, and D are of all eight samples combined. Each boxplot displays: sample median (bold line within box), interquartile range (box), minimum and maximum lengths with exception of outliers (whiskers), and likely outliers (circles).

Both the physical and chemical characteristics of microplastic contaminants can influence their fate in the environment and the impact they may have organismal health including via ingestion, the primary route for biological uptake (GESAMP, 2016). Feeding habits of organisms influence the size and shape of microplastics they ingest. Aytan et al. (2022 reported fragments are ingested by copepods more so than fibres, while other reports indicate planktivorous fish and sea cucumber ingest a higher amount of fibres (Husin et al., 2021; Jensen et al., 2019). Although the findings here are preliminary, given the similar distribution of microplastic fragments and fibres (48% vs 52%) found in the NSW subsurface seawater, it can be speculated that similar taxa inhabiting the water column will be exposed to similar level of risk regardless of the preference for one shape over the other. Smaller microplastics are considered to have higher bioavailability, i.e., more organisms are capable of microplastic ingestion, either accidentally, incidentally or intentionally. For example, copepods and other small zooplankton (Aytan et al., 2022; Sun et al., 2018; Taha et al., 2021) have been found to have ingested microplastic sizes also reported in much larger animals (Caldwell et al., 2022; Schlawinsky et al., 2022). The smaller sized microplastics found in the NSW subsurface seawater samples are thereby likely to be readily bioavailable to zooplankton, with potential for trophic transfer through the food web (Miller et al., 2020). Colour, on the other hand, is a physical characteristic of microplastics that influences primarily visual feeding biota, such as birds, turtles and fish. Higher percentages of transparent and blue coloured microplastics have been reported in wild caught fish in comparison to other colours (Jensen et al., 2019; Lopez-Martinez et al., 2021; Ory et al., 2017), although it remains to be established whether this intake is preferential, or simply a reflection of the colour profile of the contamination in the environment. Finally, the polymer profile of the samples, which reflects global reports, may influence intake. Some polymers are also more susceptible to biofouling (Agostini et al., 2021), and these biofouling organisms can emit chemistry potentially making the microplastic item more attractive to feeding organisms (Fabra et al., 2021; Feng et al., 2020). Here there was no evidence of biofouling observed suggesting the microplastics found are likely newly introduced in the environment, potentially from estuary outflows and/or wastewater outfalls. The high diversity of microplastic characteristics found in this study indicates a multitude of potential associated risks for NSW seawaters in relation to microplastic contamination but, currently, it is not known whether local fauna is susceptible to this microplastic risk. Although findings here are congruent with those in the literature, the small number of samples collected for this study does not support a robust risk assessment. Thus, there is a need to establish a baseline on microplastic contamination in NSW waterways, estuaries, coastal and offshore locales to address microplastic exposure, bioavailability and better assess risks.

5 CONCLUSIONS

Presented here is the development and validation of a stepwise protocol to process seawater samples collected by the SubCtech OceanPack™ RACE® Microplastic Sampler, an *in situ* sampling device that increases data spatial cover representativeness by allowing near-continuous subsurface seawater sampling. Seawater samples collected onto 100 µm SSSF were successfully processed using all four stepwise protocols, with each deemed suitable and effective for the retrieval of microplastics. Economic considerations established 10% KOH → KOH protocol as the protocol of choice. Its application to subsurface seawater samples collected under sail revealed all NSW waters sampled

adjacent to land outflows and outfalls contained microplastic fragments and fibres, mainly smaller than 1 mm in size, blue and transparent in colour, and polyethylene or polyester based. Nevertheless, polymer diversity was high, suggesting widespread and multifarious sources of microplastic contamination. As different tow methods have previously been used to determine microplastic concentrations in Australian waters (Jensen et al., 2019; Kroon et al., 2018a; Miller et al., 2022a; Reisser et al., 2013) it is not possible to directly compare findings here (~66 L, subsurface position, smaller 100 μm aperture) to these reports (<66 L, surface, >100 μm aperture). Compared to subsurface seawaters collected from other regions across the world, microplastic concentrations reported here indicate a high loading of microplastics in the nearshore seawaters of the Sydney region. Even though the data set was limited to eight samples over one day and confined to 100 km, findings indicate there is a large spatial variation in microplastic contamination across the sampling transect. A higher number of items were found in transect T_5, immediately southern of major estuary outflows and wastewater outfalls of Sydney, with most being microplastic fibres. It should be noted that this small dataset precludes any statement on whether estuary outflows and wastewater outfalls generate hotspots for microplastic contamination in adjacent NSW waters, but it suggests similarities between microplastics transport and the transport of estuary and wastewater plumes in the region. To further investigate this, more detailed temporal and spatial studies are needed to generate baseline information on the level of microplastic contamination in NSW waters. Both *in situ* studies and numerical simulations would be of relevance. Such information is critical to inform NSW and Australian policy and current and future management strategies to mitigate marine plastic pollution.

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