Impacts of microplastics on coastal biota and the potential for trophic transfer

By

Louise Tosetto

9 October 2015

Masters of Research (MRes) Thesis

Department of Biological Sciences

Macquarie University

Declaration

I wish to acknowledge the following assistance in the research detailed in this report:

1. Associate Professors Jane Williamson and Culum Brown for assistance with experimental design, analysis and manuscript preparation.

2. Dr Jim Lowry for assistance with identification of beachhoppers and Dr Lucia Fanini for assistance with experimental design.

3. Professor Simon George and Sarah Houlahan for undertaking GC MS analysis of microplastics and beachhoppers, and for assistance with interpreting results.

All other research described in this report is my own original work.

Louise Tosetto
9 October 2015.

Note to Examiners

This thesis was written in the format of two chapters to be submitted to two different journals:

Chapter 1: Full Research Paper in Environmental Pollution
Chapter 2. Report in Current Biology

The two chapters are formatted as per the instructions for the above journals except where Macquarie University have specified different requirements. Current Biology reports are presented with a Summary and 2,500 word Results and Discussion section. Any other information is included in the supplementary information section. I have included my methods and materials for chapter 2 at the end of the chapter rather than in a supplementary section. Environmental Pollution has no strict requirements on references as long as the style is consistent. Current Biology requires references to be cited by number in the text, however, for the purposes of examination I have followed the Harvard style of referencing throughout. Neither journal requires figures or tables at the end of the manuscript, so I have included them in the main body of the document for readability.

Acknowledgments

A huge thank you to my supervisors, Jane Williamson and Culum Brown for their excellent support, feedback and believing in this study when I had started to doubt. It's fair to say I would not have been here without you.

To all my volunteers in field work and testing; Gemma White, Jennalee Clarke, Evan Byrnes, Belinda Fabian, Penelope Carbia, Andrew Irvine, Jess Thompson, David Connolly, Rachel King and Ben Buchan, plus the numerous interns and volunteers from the biology department, couldn't have done it on my own.

Thanks to Macquarie Engineering and Technical Services (METS), especially to Walter for continual help with cutting, sawing and gluing. To Sydney Institute of Marine Science (SIMS) for the use of the facilities and the Microscopy unit at Macquarie University. A special thanks to Simon George for coming to the rescue in the final hours but more importantly to Sarah Houlahan for taking this project on with so much enthusiasm and passion it was contagious.

To my friends and family, particularly, my brother Mike and mum Penny, who have endured a year of beachhoppers, fish and plastic. Your support and availability to listen (even when you didn't understand what I was saying), was invaluable.

To the fantastic team in the MEG and BEEF labs at Macquarie University and most importantly to our writing group; Belinda, Fonti, Katie, Rich and Elise, the support, empathy and most important, the laughs, were without a doubt, one of the most important things in completing this thesis.

And lastly, but certainly not least, thanks to the beachhoppers and fish for participating. Truly, I could not have done it without you!

General Abstract

Microplastics are ubiquitous in the marine environment worldwide, and pose a physical and chemical risk to marine organisms. Their small size makes them bioavailable to a range of organisms with evidence of ingestion across the food chain. Despite an increasing body of research into microplastics, few studies have explored how consumption changes complex behaviours such as predator awareness and sociality. Furthermore, our understanding surrounding the trophic transfer of associated microplastic contaminants and the resultant effects on the food web remain largely unknown. My research assessed the impacts of microplastics on the ecology of marine biota and the potential for trophic transfer using coastal ecosystems as a model. Beachhoppers (*Platorchestia smithi)* readily ingested microplastics with subsequent alterations in behaviour. Contaminated beachhoppers were fed to Krefft's frillgobies (*Bathygobius krefftii)*, and changes in personality were assessed. There was no effect on fish personality due to an increased plastic diet suggesting trophic transfer of microplastic is not an additional exposure pathway for contaminants. This study suggests consumption of microplastics may compromise some behaviours necessary for survival. While in the short term there is no behavioural evidence of trophic transfer, further studies should seek to understand the longer-term effects of microplastics and their effect on the food web.

Chapter 1

Microplastics on our beaches: Ingestion and behavioural consequences for beachhoppers

Louise Tosetto, Culum Brown, Jane Williamson

SAEF lab, Department of Biological Sciences, Macquarie University, North Ryde, Sydney NSW 2109

Corresponding author: Louise Tosetto

***Formatted for Journal: Environmental Pollution**

ABSTRACT

Microplastics are ubiquitous in the marine environment worldwide, and pose a physical and chemical risk to marine organisms. Their small size makes them bioavailable to a range of organisms with evidence of ingestion across the food chain. Despite an increasing body of research into microplastics, few studies have explored how consumption changes complex behaviours and cognitive processes. Alterations to behaviour and cognition can lead to sub optimal conditions for individual organisms but also serve as a warning sign for wider effects on a system. This research assessed the impacts of microplastics on the ecology of marine biota using beachhoppers (*Platorchestia smithi*) as model organisms. We exposed beachhoppers to contaminated microplastics to understand effects on survival and behaviour. Beachhoppers readily ingested microplastics, and in some cases these plastics accumulated throughout the organism. Exposure tests of 72 and 120 hours showed no distinct effects of microplastic consumption on mortality. Following exposure there was a significant effect on jump height with and an increase in time to relocate shelter following disturbance. Overall, these results show that while short-term ingestion of microplastics may have no direct impact on survival of *P. smithi*, their behaviours may be compromised.

KEYWORDS: microplastics, plastic, polyethylene, behaviour, beachhopper

HIGHLIGHTS

- Polycyclic Hydrocarbons adhere to Polyethylene Microspheres
- Microplastics are readily ingested by, and can accumulate in, beachhoppers.
- Short-term consumption of microplastics has no direct effect on survival.
- Microplastic affects jump height and shelter relocation times in beachhoppers

INTRODUCTION

Plastic is a contemporary and global issue for marine environments due to its essential role in modern lifestyle and economy. Manufacture has continuously increased since the 1950s and it is estimated that between 6.4 and 8 million tons of plastic end up in the oceans every year (Allsopp et al., 2006). Deleterious effects of large plastic debris on the marine environment and wildlife have been widely reported (Derraik, 2002, Barnes et al., 2009). However, an increasing issue in our marine systems, and one that has been gaining momentum for the past decade, is contamination via small plastic fragments called microplastics (Thompson et al., 2004, Law and Thompson, 2014). Microplastics, small plastic fragments less than 2mm, pose a diverse risk to individuals, populations and communities within marine ecosystems. Microplastics are introduced to the environment directly (for example, via cosmetic beads) or indirectly through breakdown and fragmentation of larger plastic debris (Andrady, 2011). Produced from a variety of polymers for an array of uses, plastics range in densities and thus span the depth of the water column (Engler, 2012) also accumulating in benthic and coastal sediments (Law and Thompson, 2014). Such widespread distribution exposes a broad range of organisms across the food chain with consumption of microplastics demonstrated in a range of feeding guilds (Thompson et al., 2004) and across multiple trophic levels from zooplankton (Cole et al., 2013) to fish (Boerger et al., 2010) and seals (Eriksson and Burton, 2003).

In addition to the potential physical effects resulting from microplastic ingestion such as false satiation and reduced nutrition (Murray and Cowie, 2011, Cole et al., 2013), chemical effects due to associated contaminants present additional implications for marine biota. Potentially toxic additives such as phthalates, bisphenol A (BPA) and flame-retardants are incorporated into many plastics during manufacturing to increase functionality and extend the life of the plastics (Browne et al., 2008b). Furthermore, plastics are porous and thus accumulate and concentrate contaminants including polychlorinated biphenyls (PCBs), pesticides and fertilizers at high intensities from the surrounding seawater (Teuten et al., 2007). Many of the additives and the absorbed contaminants are known endocrine disruptors, carcinogens and mutagens (Lithner et al., 2009). Despite conceptual and biodynamic models simulating effects of plastic on the bioaccumulation of contaminants suggesting that plastics are negligible pathways for exposure (Koelmans, 2013, Koelmans, 2014), recent studies found that gut surfactants,

temperature and pH can all influence the rate of desorption into animals (Bakir et al., 2014). Moreover, the transfer of pollutants from microplastics to animals has been demonstrated (Ryan et al., 1988, Teuten et al., 2009, Avio et al., 2015), and it is possible that microplastics provide additional passage for contaminants to accumulate in biota.

While a wide range of organisms can ingest microplastics, our understanding of biological effects of such ingestion remains limited. Reported effects on survival, growth and fecundity are inconsistent and often found at high concentrations of microplastic exposure. Increased mortality was observed in copeopods (*Tigriopus japonicus)* but only at large dilutions of microplastics (Lee et al., 2013). An effect on growth was reported for body width of sea urchin larvae (*Tripneustes gratilla*) at the highest concentration of microplastics (Kaposi et al., 2014). Impacts on fecundity have been reported for copeopods *T. japonicus* in a two generation chronic toxicity test (Lee et al., 2013) and *Calanus helgolandicus* with a reduction in egg size (Cole et al., 2015). Changes in physiology including hepatic stress in fish (Rochman et al., 2013b), reduced immunity in lugworms (Browne et al., 2013) and formation of granulocytomas in mussels - a cellular response often indicative of pollutant exposure (von Moos et al., 2012) - have also been reported. However, a recent study on a marine isopod *Idotea emerginata* showed no significant effect of microplastics on survival, growth and fecundity following ingestion (Hämer et al., 2014). Variations in biological responses to different concentrations of microplastics advocate a more consistent approach to assessing environmentally relevant concentrations of microplastics.

In many animals, behaviour modification is often the first response to a change in conditions (Wong and Candolin, 2015), and can indicate subsequent survival and physiological issues (Scott and Sloman, 2004). In a study on shore crabs (*Carcinus maenas*) exposed to pyrene, no significant impacts were observed at the cellular or physiological level, however, a significant reduction in foraging behaviour was observed in exposed crabs (Dissanayake et al., 2010). Studies so far assessing any behavioural effects from microplastics have focused on changes to foraging rates (Besseling et al., 2012, Wegner et al., 2012, Cole et al., 2013, Wright et al., 2013), but changes to complex behaviours such as predator interactions and burrowing as well as cognitive abilities including social recognition and orientation are also essential to our understanding of the ecological impacts (Ungherese and Ugolini, 2009, Weis, 2014b). Exposing animals to more environmentally relevant concentrations and evaluating behaviours in relation to

an animal's habitat is important. Behavioural responses provide useful markers of pollution effects on individuals, potentially performing as reliable and economical indicators of sub lethal affects of pollutants (Weis, 2014a). Accordingly, there is growing emphasis in ecotoxicology on examining changes in behaviour in response to exposure to contaminants, which is far more sensitive than the standard LD50 (median lethal dose) approach (Oulton et al., 2014). Given the connection that behaviour provides between physiological and ecological processes, behavioural alterations not only indicate issues at the individual level but also serve as a warning sign for wider effects on a system (Weis, 2014b).

Microplastics accumulate in coastal areas (Browne et al., 2011, Setälä et al., 2016), and density of microplastic has been reported as high as 3% weight in highly impacted sites (Carson et al., 2011). There is also evidence for the accumulation of contaminants onto microplastics in coastal sediments (Frias et al., 2010). The supralittoral zone is an ecologically important area on sandy shores providing important connectivity between the surf and sand dunes (Ungherese et al., 2012). These zones receive large inputs of algal and seagrass wrack (Gonçalves and Marques, 2011) and are typically dominated by crustaceans such as coastal talitrid amphipods (Gonçalves et al., 2013). Coastal talitrids (Amphipoda, Talitridae) are highly mobile organisms that inhabit sediment in wave-washed beaches. They carry out an important ecological role in the function of sandy shores by commencing the decomposition processes of algae and wrack, essential for benthic sediment remineralisation processes (Dugan et al., 2011). They are also primary consumers and an important food source for birds, insects and fish (Wildish, 1988), therefore important in the transfer of energy between the different trophic levels (Griffiths et al., 1983).

Coastal talitrids have been shown to consume microplastic (Ugolini et al., 2013), and have been identified as a good bioindicator species for accumulation of chemicals within the environment (Ungherese et al., 2012). Given their life history and behaviour is well understood (Scapini, 2006), they make a useful species to study the effects of plastics. The exposed nature of sandy beaches places beachhoppers at risk of predation, furthermore, talitrids do not have a large physiological capacity to survive in such harsh environments and desiccation is another threat (Koch, 1989). Salutatory locomotion and behavioural adaptations such as well-refined hygrokinesis, the ability to locate and remain in optimal humidity are important in avoiding desiccation and predation

(Ugolini, 1996, Morritt, 1998). Any fundamental changes to the behaviour of key organisms such as coastal talitrids could have flow on effects to their environment and higher trophic levels. It is important to understand the impacts that a contemporary pollutant such as microplastics is having at the base level of the ecosystem.

This study assessed the effects of microplastics on beachhoppers. *Platorchestia smithi* (Lowry, 2012), a beachhopper found in the Sydney region of Australia was used as the study species. Given desiccation and predation are two of the biggest risks to beachhoppers, the ability to quickly relocate shelter or escape predation is key to survival. While whole organism performance capacity is important in terms of broad ecological function, prey can increase their probability of escaping a predator attack by using behaviours that enhance response times or shorten distances to the nearest refuge (Hawlena et al., 2011, McGinley et al., 2013). Given Ugolini (1996) suggests that jumping in *T. saltaor*, is an important anti-predator approach this study assesses jumping height and frequency as a proxy for fitness. It also examines cognitive processes such as orienting to suitable shelter. It is possible that microplastics may negatively impact on these behaviours, reducing the capacity of these animals to survive in their environment. Specifically we asked the following questions. 1. Do beachhoppers ingest microplastics when exposed at environmentally relevant concentrations? 2. Do beachhoppers concentrate additives from contaminated plastic? 3. Does plastic ingestion affect survival? 4. Does plastic ingestion affect behaviours important for existence in their environment?

MATERIALS AND METHODS

Preparation of microplastics

Commercial polyethylene (PE) microspheres (Cospheric UVPMS-BG, 1.004g mL^{-1} density, nominal 38-45 μm diameter, colour green) were used as proxies for microplastics in marine environments (Kaposi et al. 2014). Prior to use, the microspheres were deployed in the aquarium at Sydney Institute of Marine Science (SIMS), in Port Jackson, Australia (33°50'24"/151°15'13"). Seawater at SIMS is pumped from Port Jackson, Sydney and filtered through 100 um disc filters with all other physical parameters remaining ambient. To absorb any pollutants available in the surrounding marine environment, 40g of microspheres were placed in a banjo filter (100 x 100 mm) fitted with 22 um mesh. Water from the harbour was able to flow freely through the filter, thus exposing the microspheres to contaminants present. We contaminated the plastics for two months (Rochman et al., 2013a) after which the filter was collected and plastics dried at room temperature for three weeks prior to use in exposure experiments. Due to the small amount of microplastics used in the study, only 0.2 g could be analysed at two time periods: one when the microplastics initially obtained prior to use, and the other after three months of exposure to seawater in the field.

Contamination Analysis – PE Microplastics

Microplastics were assessed for contamination post exposure in Port Jackson. Polycyclic Aromatic Hydrocarbons (PAHs) are one of the most widespread organic pollutants in the aquatic environment (Gonçalves et al., 2008) and given PE has a high sorption capacity for PAHs in the aquatic environment (Fries and Zarfl, 2012), PE microplastics were assessed for uptake of PAHs, which was used as a proxy for a range of contaminants. Polyethylene microsphere treatment samples (0.2 g) were placed in a beaker with 30 ml of dichloromethane/hexane (1:1) and extracted using ultrasonication for 25 minutes. Samples were allowed to settle and transferred to another beaker for reduction using nitrogen blowdown then transferred to 2 ml injection vial. Samples were spiked with an internal standard (p-Terphnyl-d14) then run on GC-MS.

Study Organisms

P. smithi were collected by hand from March to July 2015 from the supralittoral zone of Forrester's Beach, NSW (33°24'37.06", 151°28'05.39"). Individuals were maintained in

damp sand and transported to Macquarie University in Sydney where they were held in plastic tubs (60cm x 40cm) with approximately 4 cm sand and a covering of the fresh kelp *Eklonia radiata.* Tubs were covered with 2 mm mesh to allow for air circulation but inhibit escape of animals. Sand was kept damp with seawater and carefully mixed every third day to maintain aeration and new wrack was provided to hoppers every week. Beachhopper collections were conducted under NSW Fisheries Scientific Collection Permit number P14/0032-1.1.

Treatment Assays

Experiments were set up in 1L glass beakers (90 mm high, 50 mm diameter). Treatment beakers consisted of 25 g sediment (~0.5 cm thick layer) covering the bottom of the beaker with 1 g microplastics (3.8 % Dry Weight (DW)) evenly mixed through, while control organisms were exposed to 26g of clean sediment (no microplastics). Sediment was obtained from beachhopper sampling locations at Forrester's Beach, thus providing a baseline for contamination given this was what the beachhoppers had previously been exposed to. Prior to placement into replicates, sand was dried at 40°C for 24 hours. The treatment level of 3.8% microplastics was slightly greater than the approximated 3% that has been reported at highly impacted sites (Carson et al. 2011) but was lower than levels in previous studies that have reported deleterious effects on organisms (Besseling et al. 2014, Graham & Thompson 2009, Wright et al. 2014). Both treatment and control beakers had 10 g of fresh *Eklonia radiata* placed on top of the sand and 10 ml of freshwater. Beakers were covered with mesh and left in ambient conditions in the laboratory with natural light. 3-5 ml of freshwater was added to the beakers every two days.

Beachhopper Ingestion & Gut Retention Time

To assess the time for beachhoppers to ingest and egest microspheres, five treatment beakers were prepared as above. Twenty-five *P. smithi* (size range 8 – 10mm) were randomly selected and five placed in each beaker. Time of exposure was recorded from when beachhoppers were placed into the beakers. As the microspheres were fluorescent green, ingestion could be observed with the naked eye. Beachhoppers were monitored hourly from the initial time of exposure until the point at which microspheres were ingested by the first individual. Beachhoppers that had ingested microspheres were transferred to 1 L beakers with 25 g uncontaminated sand and 5 g wrack, and

subsequently monitored every two hours for the passage of the microspheres through the gut.

Beachhopper Contamination Analysis

To examine if contaminants were absorbed following exposure to contaminated microplastics, experiments were setup in three, 2L beakers. The control beaker consisted of 52 g sediment from Forrester's Beach. This provided a baseline for contamination given this was what the beachhoppers had previously been exposed to. Two treatment beakers had 50 g sediment and 2 g microplastic (3.8%). 20 beachhoppers were added to each of the three beakers. Following 72 hours exposure, the beachhoppers from the control and one of the treatment beakers were collected, rinsed with distilled water and frozen in liquid nitrogen. The hoppers from the second treatment were transferred from the treatment beaker into an uncontaminated beaker containing just 52 g clean sediment to allow for egestion of microplastics. After 48 hours the beachhoppers from the second treatment were collected and frozen in liquid nitrogen. All samples were stored at -30°C.

For analysis, amphipod samples were freeze dried, ground and extracted in 5ml dichloromethane using ultrasonication for 30 minutes. Samples were allowed to settle and a 4 ml aliquot was taken. The aliquot was dried over MgSO4, filtered (GF/B), and then concentrated to approximately 100 uL by nitrogen blowdown. The samples were spiked with an internal standard (p-Terphnyl-d14) then run on GC-MS. The amount of PAH (ug g^{-1}) in each sample was calculated by taking the total area for all PAH (excluding the internal standard) using the following formula;
*Total PAH/p-Terphenyl-d14 (internal standard))*Total ug standard.*

Exposure Tests

Exposure tests assessed effects of microplastics on survival and behaviours. Control and treatment beakers were set up as described above. Five *P. smithi*, size range 8 – 10mm, were randomly selected and placed into the experiment beakers. Two exposure tests were carried out at 72 hours (n=13 and 14 in both treatment groups), survival and behavioural experiments assessing jump height, jump frequency and shelter relocation were undertaken. A further test was carried out at 120 hours (control and treatment; n=11) that only assessed survival, jump height and jump frequency. Further details for each test are provided below.

Survival

The number of live beachhoppers remaning in each beaker was recorded daily. Monitoring commenced 24 hours after the hoppers were placed in the treatments. Dead beachhoppers (typically darker in appearance and nonmotile) were removed from the treatments. A Kruskal-Wallis test of differences was undertaken on the average hoppers alive per beaker in treatment and control (R Core Team, 2013).

Assessing Individual Beachhopper Behaviour

The locomotory jumping of beachhoppers is a common behaviour that is used following disturbance to locate suitable shelter (Bracht, 1980, Morritt and Spicer, 1998). Jump frequency was therefore tested to provide a measure of activity, while exposure time, shelter location and shelter duration were used to assess differences in the likelihood for the animals to avoid predation and desiccation. Treatment and control groups (n=13) were exposed to microplastic contaminated sediment and clean sediment respectively for 72 hours. Beachhoppers were tested individually; in total 65 beachhoppers were tested from each treatment. To assess these behaviours, a test arena was created using a 5L beaker (20 cm diameter) and bright cold light overhead ensuring that the entire base of the beaker was well lit. A 100 x 50 mm piece of *E. radiata* placed on one side of the beaker and an individual beachhopper was placed into the beaker opposite the seaweed. Individuals were monitored for 90 seconds from release and behaviour was recorded using a web camera (Logitech C920) positioned directly above the beaker. Trials were recorded onto a laptop computer and scored using the behavioural program Etholog™ (Ottoni, 2000). The number of jumps as well as: (1) time exposed in the beaker, (2) time to locate algae (top or underneath), (3) time move underneath the algae and (4) time spent under shelter was recorded for each individual. If beachhoppers did not find the algae then exposure time was recorded as 90 seconds. Tests were alternated between control and treatment beakers.

To assess for differences between treatments and controls, variables were averaged for the five hoppers in each beaker and a Wilcoxon Signed Rank test of differences was undertaken on the averages. Analyses were performed in R 3.0.2 (R Core Team, 2013).

Assessing Group Beachhopper Behaviour

Throughout the shelter location experiments it was observed that beachhoppers were gregarious. They congregated under patches of algae, and when in holding beakers

sought shelter together. To assess if there were differences in group behaviour, experiments were established to assess jump height, jump frequency, and shelter relocation times following disturbance.

Jump Height & Frequency

Differences in jumping height between individuals exposed to microspheres were compared to control individuals. Height was assessed in a 2 L beaker with measurements outlined in 1 cm intervals from 0 to 18 cm. A piece of fresh *E. radiata,* 100 x 50 mm, was placed in a beaker and beachhoppers were added, allowing 2 minutes for individuals to acclimate and shelter under the algae. After two minutes the *E. radiata* was removed and the beachhoppers were given 5 puffs of air in quick succession from a lens cleaner (Daiso blower). A DLSR, Canon 650D, was set up adjacent to the beaker and trials recorded onto a SD card for future analysis. Videos were analysed frame by frame for 500 frames (20 seconds) from the time the last puff was administered using Quick Time Player 7 (version 7.6.1) with jump height and frequency recorded for all five beachhoppers.

There were two experiments conducted at 72 hours exposure. Data for the two experiments was pooled (n=27) given that there was no significant difference in the jump height or frequencies between the trials ($p > 0.05$). A further experiment was conducted at 120 hours exposure (n=11). To assess differences in mean jump height, a mixed effects model was produced to assess differences in hopping height between the two treatments taking into account the variability due to the different beakers. Fixed effect was treatment with beakers as a random effect. The mixed effects model was analysed using lme4 package (Pinheiro J, 2014) in R (R Core Team, 2013). To calculate differences in average jump frequency the total number of hops recorded over 500 frames was divided by the number of individuals in the beaker (5), to provide the average number of hops per individual. A linear model was used to assess for differences between the groups using the lm function in stats package in R (R Core Team, 2013).

Group Shelter Relocation

When algal wrack is disturbed on a sandy shore, beachhoppers need to relocate suitable shelter quickly so as to minimise the risks (biotic and abiotic) present in the surrounding environment (Morritt, 1998). Time to recolonise shelter following

disturbance was tested for control and treatment groups. Five hoppers from each beaker were placed into a clean 1 L beaker with a 100 x 50 mm piece of *E. radiata* in the centre. The beaker was placed under a cold light and individuals left for two minutes to seek shelter beneath *E. radiata.* After two minutes the macroalga was removed, the beachhoppers scattered for 30 s, and then the macroalga was replaced. The time taken for the beachhoppers to recolonise the algae and hide underneath was recorded for each individual. Trials were recorded on web camera (Logitech C920). Recolonisation was classified as when the beachhopper's entire body was underneath the macroalga. Trials ended when all beachhoppers sought shelter under the alga, or once 90 seconds had expired.

The shelter relocation experiment was undertaken twice at 72 hours exposure. Given there was no significant difference ($p < -0.025$) between the replicate experiments, the data from the two orientation experiments was pooled for analysis ($n = 27$). Differences in times for individual hoppers, as well as the average time for all hoppers to recolonise the algae, were compared between treatments using a Kruskal-Wallis test of differences in R (R Core Team, 2013).

RESULTS

PAH Analysis

Analysis of the two sets of PE microplastics found varying amounts of PAHs. No PAHs were present on the microplastics prior to deployment at SIMS. Following exposure of microplastics to seawater from Sydney Harbour, 0.007 ug g^{-1} of PAHs were present.

Ingestion and Gut Retention Time

Observations of beachhoppers demonstrated that microplastics can be ingested as part of normal feeding. Of the twenty five beachhoppers exposed to microplastics, 22 (88%) consumed them over the 48 hour duration of the experiment. In the first two hours following exposure, 18 (72%) beachhoppers consumed microspheres, the other 6 consumed them in the next 4 hours. The majority of beachhoppers egested microplastics in less than 4 hours with the remaining egested over the next 48 hours. A representative beachhopper after 72 h was placed under a digital microscope and shows the extent that these plastics can move through the animal (Figure 1).

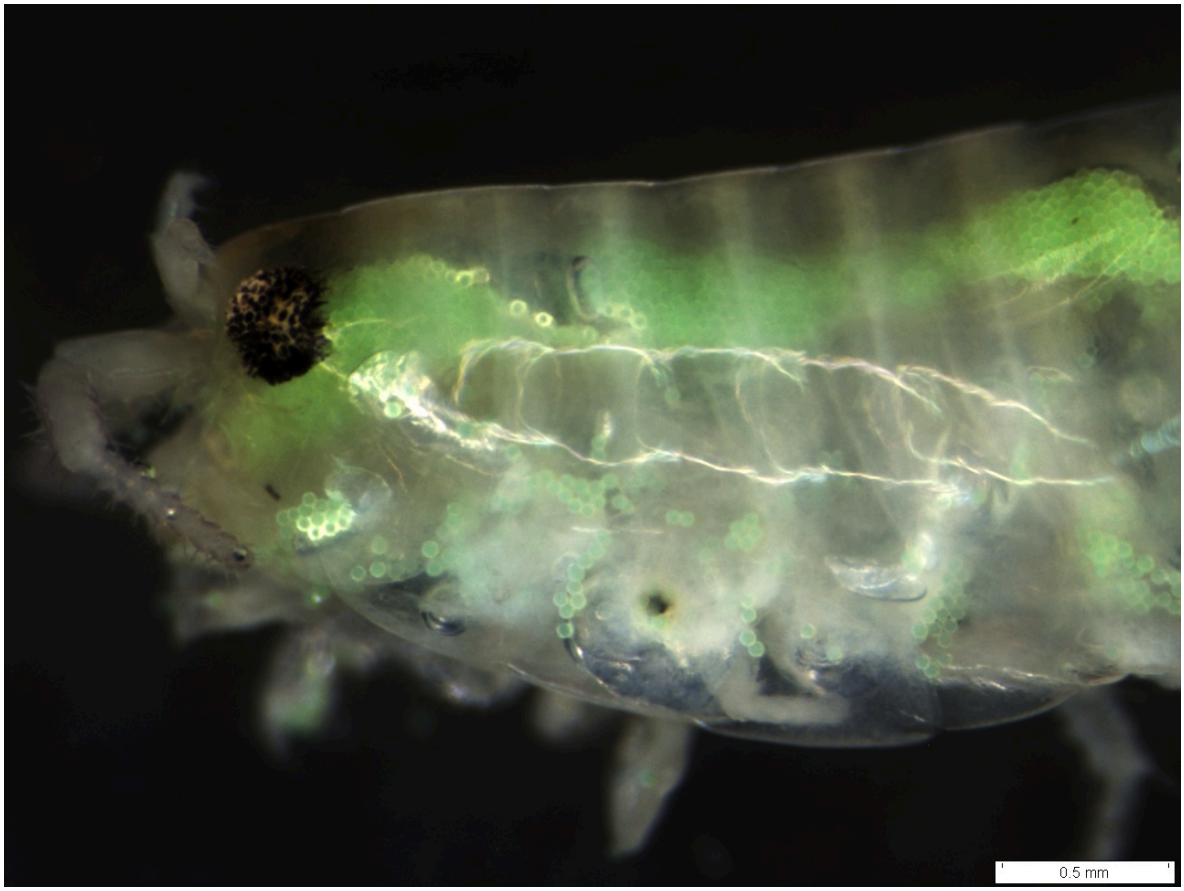

Figure 1. Beachhopper exposed to microplastics for 72 hours. The green circles are the microspheres that have moved through the gut and transferred throughout the body cavity.

Beachhopper Contamination

After 72 hours exposure, beachhoppers in the control group contained 2.34 ug g^{-1} PAH, while those in the treatment group had 3.09 ug g^{-1}, an increase of 0.74 ug g^{-1}. However, following 48 hours post treatment, contamination levels had dropped to concentrations on par with those in the control group (2.21 ug g^{-1}) suggesting there was little or no residual contamination of PAHs following egestion of microplastics.

Individual Beachhopper Behaviour

Ingestion of microplastics did not alter the use of macroalgae as a refuge. Following 72 hours of exposure there were no significant differences in the time exposed (W = 95, P = 0.867), the time taken to find the macroalgae (W = 92, P = 0.981), the time to move undercover (W = 81.5, P = 0.662) or the time spent underneath the algae (W = 81, P = 0.650) for beachhoppers in each treatment. Additionally, there was no effect of microplastic ingestion on jump frequency (W = 110, P = 0.369).

Survival

The pooled data for the 72-hour exposure tests showed very little difference in survival between control and treatment groups. In the 120-hour exposure experiment there were significant differences in the control and treatment groups at 72 hours (W = 150.5, N = 27, P <0.001), 96 hours (W = 132, N = 27, P = 0.014) and 144 hours (W = 120.5, N = 27, P = 0.047). While the treatment group had lower survival rates at 120 hours, this difference was not significant (W = 119, N = 27, P = 0.072). The total survival time was the same for both 72 and 120 hour experiments with no surviving hoppers after eight days (Figure 2).

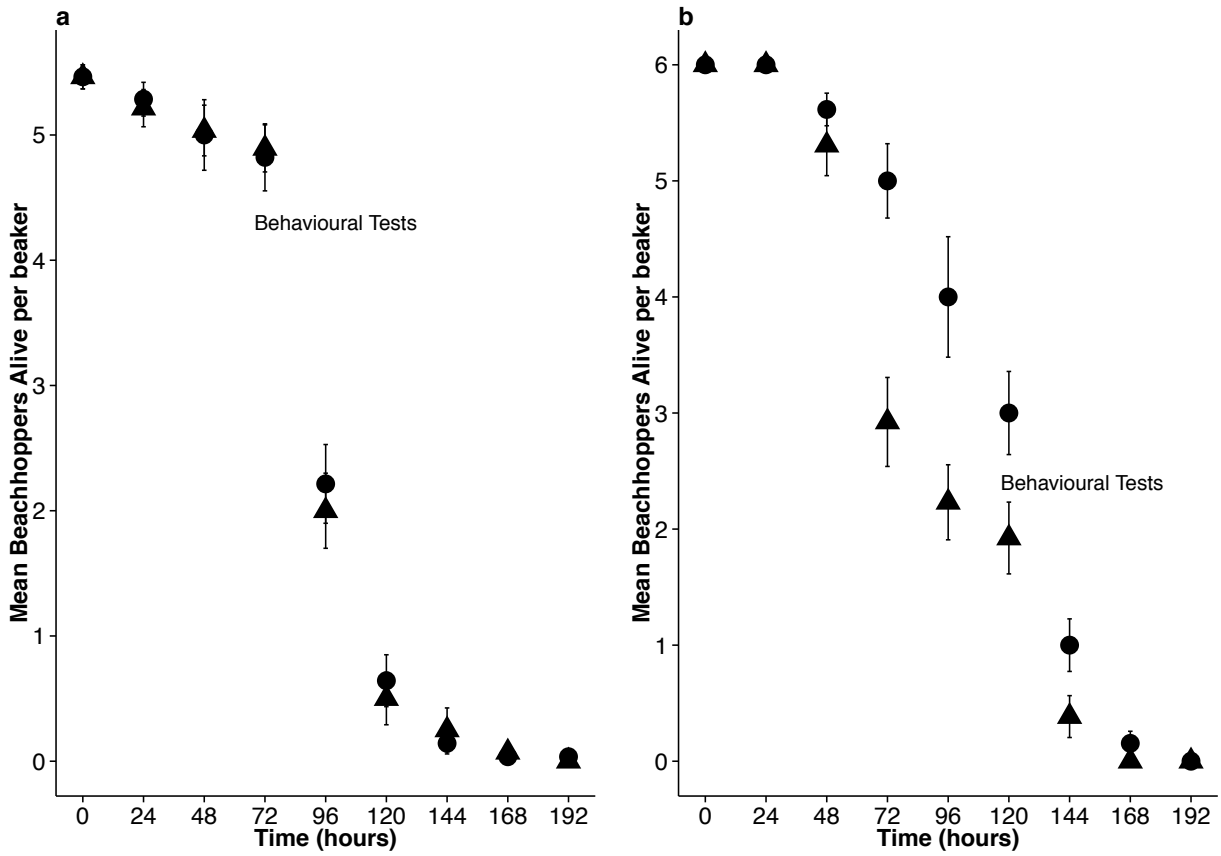

Figure 2. Mean survival (±SE) for beachhoppers every 24 hours for the 72 hour (**a**) and 120 hour (**b**) exposure tests. 72 hour test was run twice and data pooled (n = 27), 120 hour tests run once (n=11). Circles represent control group, triangles represent treatment group. Asterisks represent significance: **P* < 0.05, ***P* < 0.01.

Jump Height & Frequency

While not significant, there was a variance in jump height after 72 hours, with control beachhoppers jumping slightly higher than those in the pooled plastic treatment (t = -1.86, df = 42, P = 0.069). No significant difference between the number of jumps taken over twenty seconds between the pooled control and treatment groups was observed (t = 0.14, df = 51, P = 0.887) (Figure 3).

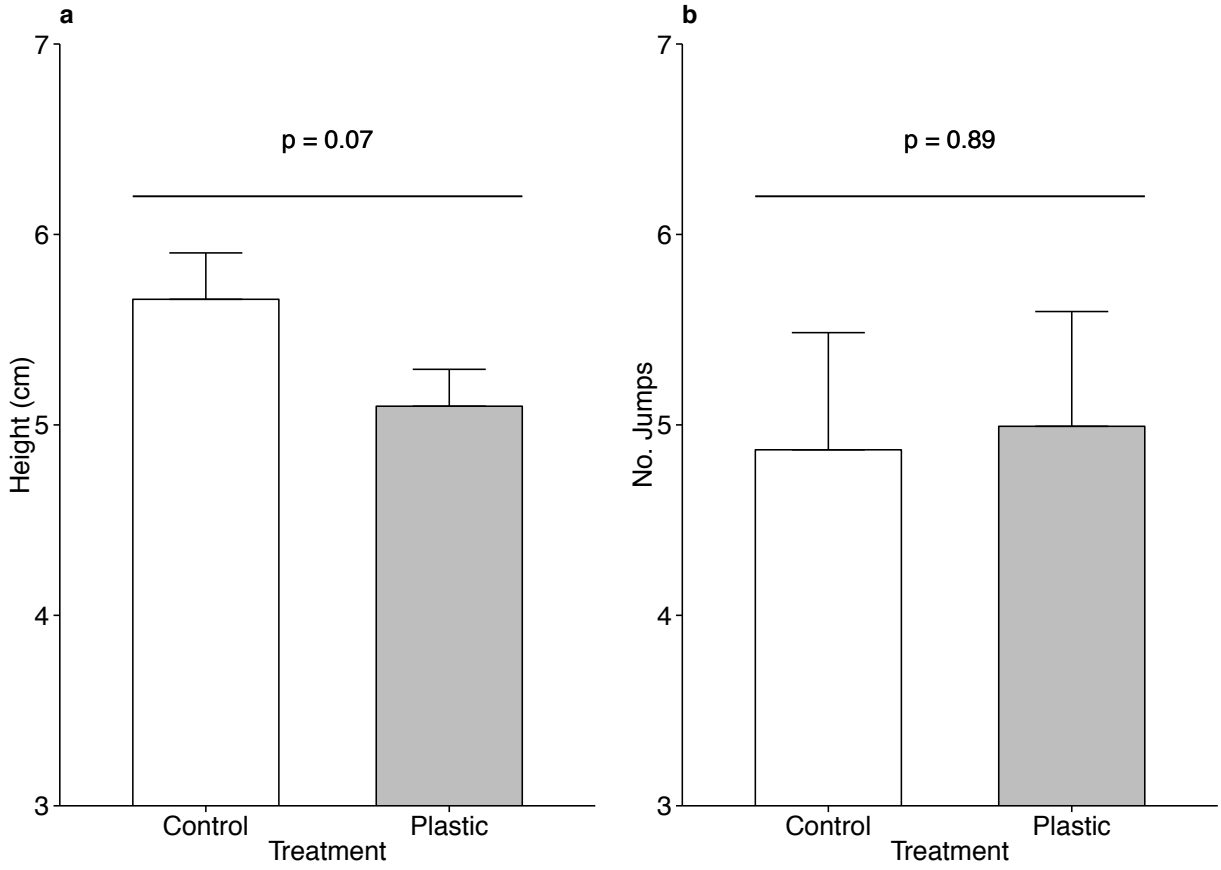

Figure 3. Mean (+SE) Jump Height (a) and Frequency (b) for beachhoppers after 72 hours exposure (n = 27). Control groups indicated by white bars, treatment groups indicated by grey bars.

Following an exposure period of 120 hours, the jumping height of beachhoppers exposed to microplastics was significantly lower than those in the control group. The linear mixed effects model demonstrated that hoppers exposed to microplastics hopped 1.6 cm lower than those in the control group (t = -2.79, df = 18, P = 0.012). The mean number of jumps per beachhopper did not differ significantly between the treatment and control groups (t = 0.33, df = 22, P = 0.744) (Figure 4).

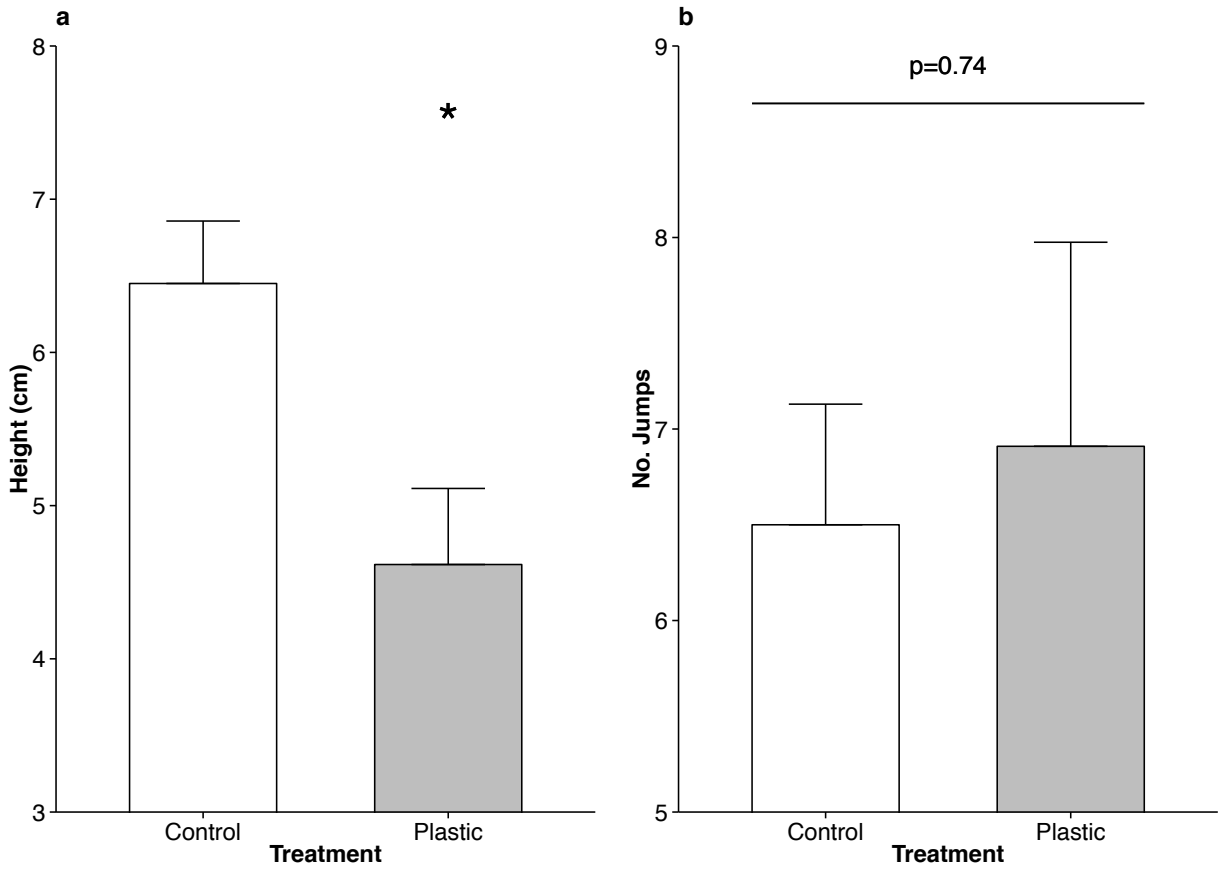

Figure 4. Mean (+SE) Jump Height (a) and Frequency (b) for beachhoppers after 120 hours exposure (n = 11). Control groups indicated by white bars, treatment groups indicated by grey bars. Asterisks represent significance: * $P < 0.05$

Group Shelter Relocation

Individual beachhoppers exposed to microplastics took five to seven seconds longer, on average, than those in the control treatment, but these differences were not statistically significant (Table 1). Overall, beachhoppers in the microplastic treatment took 6.5 seconds longer than those in the control group to relocate the algae following disturbance ($t = 1.02$, $df = 49$, $P = 0.313$) (Figure 5).

Table 1. Summary statistics from combined shelter relocation experiments. The average time (+SE) taken for each of the five beachhoppers from control and treatment groups to relocate shelter following disturbance is provided.

		Mean Time (s)	SE (s)	X^2	P-Value
First Hopper	Control	5.5	0.8	0.17	0.68
	Microplastic	10.3	19.5		
Second Hopper	Control	13.4	13.3	0.35	0.55
	Microplastic	18.3	24.8		
Third Hopper	Control	25.5	2.7	0.06	0.81
	Microplastic	30.8	32.6		
Fourth Hopper	Control	39.5	5.6	0.04	0.84
	Microplastic	48.9	7.3		
All Hoppers	Control	66.1	7.6	0.55	0.46
	Microplastic	73.9	7.0		

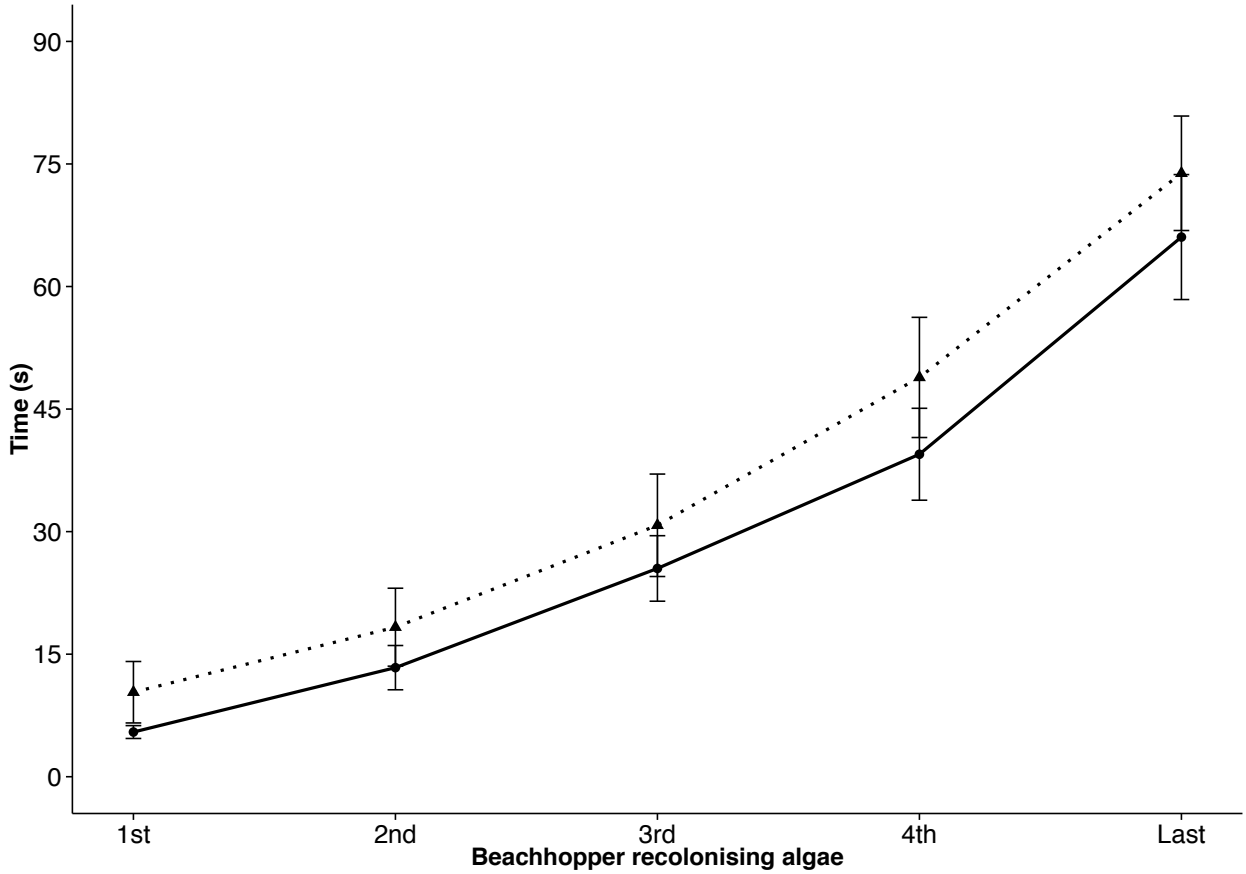

Figure 5. Mean (+ SE) differences in shelter relocation times between beachhoppers in pooled control (solid line) and treatment groups (dotted line) after 72 hours exposure (n = 27)

DISCUSSION

Our results demonstrate that microplastics are ingested by, and can accumulate in beachhoppers. The microplastics that had been placed in the marine environment had an increased concentration of PAHs compared to the original microplastics suggesting that they readily absorb contaminants from seawater. A spike in PAHs was observed in the beachhoppers following 72 hours exposure to microplastics, however, 48 hours after transfer to an uncontaminated sediment, the levels of PAHs dropped to background levels suggesting there was no residual PAHs in the hoppers following egestion. No overall differences in survival were observed in either of the exposure experiments but exposure to microplastics increased mortality at multiple time points in the 120 hour exposure treatment. Consumption of microplastics resulted in decreased jump height, potentially resulting from an increase in beachhopper weight. There were no differences between treatments in the time taken for beachhoppers to relocate shelter.

Rapid ingestion rates exhibited in the laboratory suggest that beachhoppers will readily consume microplastics if present in the surrounding environment. Microplastics were tracked through the beachhopper gut and while most were egested within 72 hours, some did transfer and accumulate in the gills and cacae of the animal. Previous studies on European sand hoppers, *Talitrus saltator* (Ugolini et al., 2013) and marine isopods *I. emarginata* (Hämer et al., 2014) found continual egestion of plastics in comparable timeframes with no reported accumulation in the animals. In mussels (*Mytilus edulis*), microplastics were found to translocate from the gut to the circulatory system where they persisted for up to 48 days (Browne et al., 2008a). The findings in this study showing that microplastics can translocate in *P. smithi,* suggest that retention timeframes may be extended in natural environments.

PAHs absorbed to microplastic spheres following two months exposure to seawater from Port Jackson. The concentration of 0.007ug g^{-1} is at the lower end of what has been reported worldwide where PAHs range from 0.001 to 9.3 ug g^{-1}, the highest concentrations in industrialised and urban areas (Hirai et al., 2011). The microplastics used in this study were well preserved. Given weathered and degraded plastics have increased capacity for pollutant absorption (Endo et al., 2005, Ogata et al., 2009) it is possible that the PAHs quantified in this study are lower than what would be found under natural circumstances. Following 72 hours exposure to contaminated

microplastics there was an increase in the amount of PAHs in beachhoppers, however, there were little, or no residual PAHs following egestion of microplastics. A recent study assessing absorption of the PAH pyrene from PE to mussels following six days exposure reported an increase of 0.145 um g^{-1} (Avio et al., 2015). Whether this was residual contamination or due to the presence of internal plastics is unclear as there is no mention of a transfer to an uncontaminated diet prior to analysis. Although contaminant analysis in this study focused only on the uptake of PAHs, previous studies have shown PEs to also absorb PCBs and PBDEs (Endo et al., 2005, Mizukawa et al., 2009, Ogata et al., 2009). Given high concentrations of heavy metals, organochlorine pesticides and PCBs have been reported in Port Jackson sediments (Birch et al. 2007), it is possible that other contaminants also had an impact on the beachhoppers. However, the aim of our study was simply to see the impact of beachhoppers on PAH-contaminated microspheres and not to quantify the complete type and amount of adherence. More research is needed with replicate samples, sites and times of deployment to rigorously quantify absolute concentrations of PAH adherence and absorption rates.

Beachhopper overall survival was not greatly affected by exposure to microplastics but there were sub-lethal behavioural effects observed at both time periods. Although there were some significant differences in the 120 hour exposure test at days three, four and six, there were no significant differences in survival at the point the behavioural trials were undertaken for either 72 or 120 hour tests. Exposure to microplastics affected the jump height of beachhoppers in behavioural experiments. After 72 hours exposure, individuals exposed to microplastics jumped 10 % lower than those in the control group. When exposed for 120 hours there was a significant effect of microplastics with those in the treatment group jumping 28 % lower than those in the control. The corresponding increase in weight gain at 120 hours indicates internal microplastics may have affected the jumping ability of the beachhoppers. Interestingly, there was no difference in jump frequency between treatments with individuals averaging 5 & 6.7 jumps over 20 seconds for 72 and 120 hour exposure tests respectively. This was comparable with Ugolini (1996) reporting that following shelter disturbance, *T. saltator* individuals jumped on average 4.8 times over 20 seconds. These findings suggest that a disturbance response may be similar for talitrids and that consumption of microplastics do not have a significant impact on this behaviour. Studies assessing shelter relocation after 72 hours found a trend in the time to relocate refuge following disturbance, with hoppers in microplastic treatments taking six seconds longer, on average, than those in

the control. The differences in behaviours observed at these time points supports the idea that sub-lethal effects of microplastics and associated contaminants may not be observed when assessing survival only. Studies so far have been relatively short term with equally short observation timeframes, and longer-term effects of microplastics on survival of organisms should not be ruled out.

Reductions in beachhopper jump height may be due to the physical structure of the ingested microplastics in addition to their role in contamination. The increase in weight resulting from microplastic consumption was a likely contributor to the decrease in height jumped following disturbance. However, reduced feeding activity due to false satiation or physical blockages with a subsequent reduction in available energy for growth and metabolism may also be a driver. Following consumption of microfibers shore crabs, *C. maenas,* displayed reduced food consumption with a significant reduction in energy available for growth (Watts et al. 2015.). Likewise in the freshwater benthic invertebrate, *Hyalella azteca*, microplastics had extended residence times in the gut with a decrease in growth and reproduction (Au et al. 2015). Gut residence times for microplastics were also higher when lugworms were exposed to high concentrations of PVC thus implying that low nutrient particles are being retained and subjected to extensive digestion, again, a highly energetic costly process (Wegner et al., 2012). Alterations to feeding and gut residence times place energetic demands on organisms that may subsequently reduce primary metabolism and respiration (Weis 2014), thus affecting an organism's ability to carry out key ecological functions.

While the time taken to relocate shelter did not differ between treatments, there was an observable trend suggesting that after longer exposure periods there may be an effect in the time for contaminated beachhoppers to relocate shelter. It has been demonstrated that amphipods possess a number of cognitive processes that may influence the ability to relocate shelter. Moreover, coastal talitrids are known to be gregarious, which may also be indicative of social interactions (Beermann et al., 2015). The gregarious nature of talitrids appears to be important in regulating activity and antipredator tactics (Ayari et al., 2015a). Variations in individual body clocks and locomotor activity rhythms stabilise when in groups due to the influence of other individuals (Bregazzi and Naylor, 1972, Ayari et al., 2015c) and this social synchronisation is thought to be important for individual survival (Ayari et al., 2015a). While amphipods have not been tested for true individual recognition, it is likely they have class-level recognition (definitions provided

in Gherardi et al. 2012). Reports suggest that chemical cues, such as molt hormones, important for reproductive interactions, are the most dominant channel of communication between conspecifics in amphipods (Thiel, 2011). Pollutants have been shown to disrupt chemical cues and hormones responsible for reproductive function (Rodríguez et al., 2007), that may in turn also affect the ability for amphipods to identify conspecifics (Beermann et al., 2015). It is possible that increased contamination via persistent microplastic ingestion may alter recognition processes amongst beachhoppers.

Desiccation and predation are the two greatest risks that coastal talitrids face in their harsh environment (Defeo and McLachlan, 2005). A humid microclimate of ~85-90% relative humidity is optimal for talitrids (Morritt, 1998) and the ability for these animals to facilitate movement away from desiccating environments is key to survival (Morritt and Spicer, 1998). To maintain and relocate suitable microclimates, talitrids are thought to use various orientation mechanisms, with solar orientation being key in locating the damp belt of sand that they inhabit during the day (Ugolini, 1996, Ungherese and Ugolini, 2009). The ability for sandhopper, *T. saltator*, to use solar orientation has been shown to be affected by metals present in seawater (Ungherese and Ugolini, 2009). Likewise trace metal contamination can affect the period and diurnal distribution of sandhopper locomotor activity (Ugolini et al., 2012). Further research is required to assess if microplastics and associated contaminants are having similar effects on behavioural and cognitive processes. To date, how pollutants may affect crustacean research has largely focussed on the effects of endocrine-disrupting compounds (EDCs) on endocrine-regulated processes such as growth, moulting and sexual development (Rodriguez et al. 2007, LeBlanc 2007). Given neurohormones regulate many behavioural and physiological processes in crustaceans such as tidal rhythmicity, locomotion, a number of metabolic functions such as water and ionic balance, growth and digestion (Pinder et al., 1999) it is possible that consumption of microplastics have wide-ranging effects. Findings in this study suggest that the ability and speed to relocate to favourable environments can be compromised. Impacts on group behaviour, whether altering relocation strategy or activity levels may have important effects on a local population.

Whether the observed alterations to behaviour of beachhoppers are due to associated contaminants or to the physical nature of the ingested microplastics is not clear. The

effects that pollutants have on aquatic ecosystems and associated biota across the world have been widely reviewed (Clotfelter et al., 2004, Islam and Tanaka, 2004, Zala and Penn, 2004). Trace metals can absorb to microplastics (Holmes et al., 2014), as can persistent organic pollutants (POPs) (Endo et al., 2005, Teuten et al., 2007). If and how these contaminants can desorb from microplastics is unclear and is still debated (Gouin, 2011, Koelmans, 2013, Bakir et al., 2014, Koelmans, 2014). In a study examining PBDE uptake by amphipods in the presence and absence of microplastics, microplastics significantly reduced the uptake of PBDEs in amphipods when compared with unabsorbed free chemicals (Chua et al., 2014). However, Avio et al. (2015) provided evidence that pyrene that had adhered to polyethylene and polystyrene desorbed into the mussels and correspondingly increased in tissues, demonstrating that microplastics can be a vector for the transfer of contaminants. Regardless of whether microplastics are a significant exposure pathway for contaminants, it is likely they are providing at least a secondary conduit to what animals are already exposed to.

Results from the present study cannot be merely extrapolated to field scenarios. This was a short-term study using only one type of plastic polymer. Polymer type, weathering and plastic colour can influence the contaminants and concentrations adhered to the plastics (Endo et al., 2005). Furthermore, we know that biological responses increase significantly with exposure time (Von moos et al. 2012). While there was an increase in PAHs following exposure, there were no discernable PAHs remaining in beachhoppers following egestion of microplastics. Nevertheless, given the range of contaminants that have been demonstrated to adhere to polyethylene microplastics, other pollutants cannot be ruled out. Furthermore, undertaking longer term studies and assessing effects in situ is necessary to understand impacts on the wider system. Assessment of the wider scope was beyond the scope of this study.

This study has demonstrated that, while short-term ingestion of microplastics have little direct impact on the survival of *P. smithi*, their behaviour may be compromised. At this stage it is not clear if this change in behaviour is due to the physical or chemical effects of microplastic ingestion. It is abundantly clear, however, that behavioural assays are more sensitive than looking solely at mortality. Changes in behaviour can lead to reduced fitness such as reduced nutrition, an accumulation of contaminants within the organism, and a reduction in the ability of individuals to respond to various biotic and

abiotic cues (eg predators or desiccation). Further research is needed to understand the implications of these results in realistic field settings.

REFERENCES

Allsopp, M., Walters, A., Santillo, D. & Johnston, P. 2006. *Plastic debris in the world's oceans,* Greenpeace, Amsterdam.

Andrady, A. L. 2011. Microplastics in the marine environment. *Marine Pollution Bulletin,* 62**,** 1596-1605.

Avio, C. G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., D'errico, G., Pauletto, M., Bargelloni, L. & Regoli, F. 2015. Pollutants bioavailability and toxicological risk from microplastics to marine mussels. *Environmental Pollution,* 198**,** 211-222.

Ayari, A., Jelassi, R., Ghemari, C. & Nasri-Ammar, K. 2015a. Effect of age, sex, and mutual interaction on the locomotor behavior of *Orchestia gammarellus* in the supralittoral zone of Ghar El Melh lagoon (Bizerte, Tunisia). *Biological Rhythm Research,* 46**,** 703-714.

Ayari, A., Jelassi, R., Ghemari, C. & Nasri-Ammar, K. 2015c. Locomotor activity patterns of two sympatric species *Orchestia montagui* and *Orchestia gammarellus* (Crustacea, Amphipoda). *Biological Rhythm Research,* 46**,** 863-871.

Bakir, A., Rowland, S. J. & Thompson, R. C. 2014. Enhanced desorption of persistent organic pollutants from microplastics under simulated physiological conditions. *Environmental Pollution,* 185**,** 16-23.

Barnes, D. K., Galgani, F., Thompson, R. C. & Barlaz, M. 2009. Accumulation and fragmentation of plastic debris in global environments. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 364**,** 1985-1998.

Beermann, J., Dick, J. T. A. & Thiel, M. 2015. Social Recognition in Amphipods: An Overview. *In:* AQUILONI, L. & TRICARICO, E. (eds.) *Social Recognition in Invertebrates: The Knowns and the Unknowns.* Cham: Springer International Publishing.

Besseling, E., Wegner, A., Foekema, E. M., Van Den Heuvel-Greve, M. J. & Koelmans, A. A. 2012. Effects of microplastic on fitness and PCB bioaccumulation by the lugworm *Arenicola marina Environmental Science & Technology,* 47**,** 593-600.

Boerger, C. M., Lattin, G. L., Moore, S. L. & Moore, C. J. 2010. Plastic ingestion by planktivorous fishes in the North Pacific Central Gyre. *Marine Pollution Bulletin,* 60**,** 2275-2278.

Bracht, G. 1980. The jump of *Orchestia cavimana* Heller, 1865 (Crustacea, Amphipoda, Talitridae). *Experientia,* 36**,** 56-57.

Bregazzi, P. & Naylor, E. 1972. The locomotor activity rhythm of *Talitrus saltator* (Montagu)(Crustacea, Amphipoda). *Journal of Experimental Biology,* 57**,** 375-391.

Browne, M. A., Crump, P., Niven, S. J., Teuten, E., Tonkin, A., Galloway, T. & Thompson, R. 2011. Accumulation of microplastic on shorelines woldwide: sources and sinks. *Environmental Science & Technology,* 45**,** 9175-9179.

Browne, M. A., Dissanayake, A., Galloway, T. S., Lowe, D. M. & Thompson, R. C. 2008a. Ingested microscopic plastic translocates to the circulatory system of the mussel, Mytilus edulis (L.). *Environmental Science & Technology,* 42**,** 5026-5031.

Browne, M. A., Galloway, T. & Thompson, R. 2008b. Microplastic—an emerging contaminant of potential concern? *Integrated Environmental Assessment and Management,* 3**,** 559-561.

Browne, M. A., Niven, S. J., Galloway, T. S., Rowland, S. J. & Thompson, R. C. 2013. Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity. *Current Biology,* 23**,** 2388-2392.

Carson, H. S., Colbert, S. L., Kaylor, M. J. & Mcdermid, K. J. 2011. Small plastic debris changes water movement and heat transfer through beach sediments. *Marine Pollution Bulletin,* 62**,** 1708-1713.

Chua, E., Shimeta, J., Nugegoda, D., Morrison, P. D. & Clarke, B. O. 2014. Assimilation of Polybrominated Diphenyl Ethers from Microplastics by the Marine Amphipod, *Allorchestes Compressa*. *Environmental Science & Technology,* 48**,** 8127-8134.

Clotfelter, E. D., Bell, A. M. & Levering, K. R. 2004. The role of animal behaviour in the study of endocrine-disrupting chemicals. *Animal Behaviour,* 68**,** 665-676.

Cole, M., Lindeque, P., Fileman, E., Halsband, C. & Galloway, T. S. 2015. The Impact of Polystyrene Microplastics on Feeding, Function and Fecundity in the Marine Copepod *Calanus helgolandicus*. *Environmental Science & Technology,* 49**,** 1130-1137.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J. & Galloway, T. S. 2013. Microplastic ingestion by zooplankton. *Environmental Science & Technology,* 47**,** 6646-6655.

Defeo, O. & Mclachlan, A. 2005. Patterns, processes and regulatory mechanisms in sandy beach macrofauna: a multi-scale analysis. *Marine Ecology Progress Series,* 295**,** 1-20.

Derraik, J. G. B. 2002. The pollution of the marine environment by plastic debris: a review. *Marine Pollution Bulletin,* 44**,** 842-852.

Dissanayake, A., Piggott, C., Baldwin, C. & Sloman, K. A. 2010. Elucidating cellular and behavioural effects of contaminant impact (polycyclic aromatic hydrocarbons, PAHs) in both laboratory-exposed and field-collected shore crabs, *Carcinus maenas* (Crustacea: Decapoda). *Marine Environmental Research,* 70**,** 368-373.

Dugan, J. E., Hubbard, D. M., Page, H. M. & Schimel, J. P. 2011. Marine macrophyte wrack inputs and dissolved nutrients in beach sands. *Estuaries and Coasts,* 34**,** 839-850.

Endo, S., Takizawa, R., Okuda, K., Takada, H., Chiba, K., Kanehiro, H., Ogi, H., Yamashita, R. & Date, T. 2005. Concentration of polychlorinated biphenyls (PCBs) in beached resin pellets: variability among individual particles and regional differences. *Marine Pollution Bulletin,* 50**,** 1103-1114.

Engler, R. E. 2012. The Complex Interaction between Marine Debris and Toxic Chemicals in the Ocean. *Environmental Science & Technology,* 46**,** 12302-12315.

Eriksson, C. & Burton, H. 2003. Origins and biological accumulation of small plastic particles in fur seals from Macquarie Island. *AMBIO: A Journal of the Human Environment,* 32**,** 380-384.

Frias, J., Sobral, P. & Ferreira, A. 2010. Organic pollutants in microplastics from two beaches of the Portuguese coast. *Marine Pollution Bulletin,* 60**,** 1988-1992.

Fries, E. & Zarfl, C. 2012. Sorption of polycyclic aromatic hydrocarbons (PAHs) to low and high density polyethylene (PE). *Environmental Science and Pollution Research,* 19**,** 1296-1304.

Gherardi, F., Aquiloni, L. & Tricarico, E. 2012. Revisiting social recognition systems in invertebrates. *Animal cognition,* 15**,** 745-762.

Gonçalves, R., Scholze, M., Ferreira, A. M., Martins, M. & Correia, A. D. 2008. The joint effect of polycyclic aromatic hydrocarbons on fish behavior. *Environmental Research,* 108**,** 205-213.

Gonçalves, S. C., Anastácio, P. M. & Marques, J. C. 2013. Talitrid and Tylid crustaceans bioecology as a tool to monitor and assess sandy beaches' ecological quality condition. *Ecological Indicators,* 29**,** 549-557.

Gonçalves, S. C. & Marques, J. C. 2011. The effects of season and wrack subsidy on the community functioning of exposed sandy beaches. *Estuarine, Coastal and Shelf Science,* 95**,** 165-177.

Gouin, T. 2011. A Thermodynamic Approach for Assessing the Environmental Exposure of Chemicals Absorbed to Microplastic. *Environmental Science & Technology,* 45**,** 1466-1472.

Griffiths, C. L., Stenton-Dozey, J. M. E. & Koop, K. 1983. Kelp Wrack and the Flow of Energy through a Sandy Beach Ecosystem. *In:* MCLACHLAN, A. & ERASMUS, T. (eds.) *Sandy Beaches as Ecosystems: Based on the Proceedings of the First International Symposium on Sandy Beaches, held in Port Elizabeth, South Africa, 17–21 January 1983.* Dordrecht: Springer Netherlands.

HäMer, J., Gutow, L., KöHler, A. & Saborowski, R. 2014. Fate of Microplastics in the Marine Isopod *Idotea emarginata*. *Environmental Science & Technology,* 48**,** 13451-13458.

Hawlena, D., Kress, H., Dufresne, E. R. & Schmitz, O. J. 2011. Grasshoppers alter jumping biomechanics to enhance escape performance under chronic risk of spider predation. *Functional Ecology,* 25**,** 279-288.

Hirai, H., Takada, H., Ogata, Y., Yamashita, R., Mizukawa, K., Saha, M., Kwan, C., Moore, C., Gray, H. & Laursen, D. 2011. Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. *Marine Pollution Bulletin,* 62**,** 1683-1692.

Holmes, L. A., Turner, A. & Thompson, R. C. 2014. Interactions between trace metals and plastic production pellets under estuarine conditions. *Marine Chemistry,* 167**,** 25-32.

Islam, S. & Tanaka, M. 2004. Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis. *Marine Pollution Bulletin,* 48**,** 624-649.

Kaposi, K. L., Mos, B., Kelaher, B. P. & Dworjanyn, S. A. 2014. Ingestion of microplastic has limited impact on a marine larva. *Environmental Science & Technology,* 48**,** 1638-1645.

Koch, H. 1989. The effect of tidal inundation on the activity and behavior of the supralittoral talitrid amphipod *Traskorchestia traskiana* (Stimpson, 1857). *Crustaceana,* 57**,** 295-303.

Koelmans, A. A. 2013. Plastic as a Carrier of POPs to Aquatic Organisms: A Model Analysis. *Environmental Science & Technology,* 47**,** 7812-7820.

Koelmans, A. A. 2014. Leaching of plastic additives to marine organisms. *Environmental Pollution,* 187**,** 49-54.

Law, K. L. & Thompson, R. C. 2014. Microplastics in the seas. *Science,* 345**,** 2.

Lee, K.-W., Shim, W. J., Kwon, O. Y. & Kang, J.-H. 2013. Size-dependent effects of micro polystyrene particles in the marine copepod *Tigriopus japonicus*. *Environmental Science & Technology,* 47**,** 11278-11283.

Lithner, D., Damberg, J., Dave, G. & Larsson, Å. 2009. Leachates from plastic consumer products–Screening for toxicity with *Daphnia magna*. *Chemosphere,* 74**,** 1195-1200.

Lowry, J. 2012. Talitrid amphipods from ocean beaches along the New South Wales coast of Australia (Amphipoda, Talitridae). *Zootaxa,* 3575**,** 1-26.

Mcginley, R. H., Prenter, J. & Taylor, P. W. 2013. Whole‐organism performance in a jumping spider, Servaea incana (Araneae: Salticidae): links with morphology and between performance traits. *Biological Journal of the Linnean Society,* 110**,** 644-657.

Mizukawa, K., Takada, H., Takeuchi, I., Ikemoto, T., Omori, K. & Tsuchiya, K. 2009. Bioconcentration and biomagnification of polybrominated diphenyl ethers (PBDEs) through lower-trophic-level coastal marine food web. *Marine Pollution Bulletin,* 58**,** 1217-1224.

Morritt, D. 1998. Hygrokinetic Responses of Talitrid Amphipods. *Journal of Crustacean Biology,* 18**,** 25-35.

Morritt, D. & Spicer, J. I. 1998. The physiological ecology of talitrid amphipods: an update. *Canadian Journal of Zoology,* 76**,** 1965-1982.

Murray, F. & Cowie, P. R. 2011. Plastic contamination in the decapod crustacean *Nephrops norvegicus* (Linnaeus, 1758). *Marine Pollution Bulletin,* 62**,** 1207-1217.

Ogata, Y., Takada, H., Mizukawa, K., Hirai, H., Iwasa, S., Endo, S., Mato, Y., Saha, M., Okuda, K. & Nakashima, A. 2009. International Pellet Watch: Global monitoring of persistent organic pollutants (POPs) in coastal waters. 1. Initial phase data on PCBs, DDTs, and HCHs. *Marine Pollution Bulletin,* 58**,** 1437-1446.

Ottoni, E. B. 2000. Etholog 2.2 - A tool for the transcription and timing of behavior observation sessions. *Behaviour Research Methods, Instruments & Computers,* 32**,** 4.

Oulton, L. J., Taylor, M. P., Hose, G. C. & Brown, C. 2014. Sublethal toxicity of untreated and treated stormwater Zn concentrations on the foraging behaviour of *Paratya australiensis* (Decapoda: Atyidae). *Ecotoxicology,* 23**,** 1022-1029.

Pinder, L. C. V., Pottinger, T. G., Billinghurst, Z. & Depledge, M. H. 1999. *Endocrine Function in Aquatic Invertebrates and Evidence for Disruption by Environmental Pollutants,* ENVIRONMENT AGENCY, Bristol

Pinheiro J, B. D., Debroy S, Sarkar D, R Core Team, 2014, nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-117,

R Core Team, 2013, R: A language and environment for statistical computing. , R Foundation for Statistical Computing, Vienna, Austria.

Rochman, C. M., Hoh, E., Kurobe, T. & Teh, S. J. 2013a. Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. *Scientific reports,* 3.

Rochman, C. M., Hoh, E., Kurobe, T. & Teh, S. J. 2013b. Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. *Scientific Reports,* 3**,** 3263.

Rodríguez, E. M., Medesani, D. A. & Fingerman, M. 2007. Endocrine disruption in crustaceans due to pollutants: a review. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 146**,** 661-671.

Ryan, P., Connell, A. & Gardner, B. 1988. Plastic ingestion and PCBs in seabirds: is there a relationship? *Marine Pollution Bulletin,* 19**,** 174-176.

Scapini, F. 2006. Keynote papers on sandhopper orientation and navigation. *Marine and Freshwater Behaviour and Physiology,* 39**,** 73-85.

Scott, G. R. & Sloman, K. A. 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. *Aquatic Toxicology,* 68**,** 369-392.

Setälä, O., Norkko, J. & Lehtiniemi, M. 2016. Feeding type affects microplastic ingestion in a coastal invertebrate community. *Marine pollution bulletin,* 102**,** 95-101.

Teuten, E. L., Rowland, S. J., Galloway, T. S. & Thompson, R. C. 2007. Potential for plastics to transport hydrophobic contaminants. *Environmental Science & Technology,* 41**,** 7759-7764.

Teuten, E. L., Saquing, J. M., Knappe, D. R., Barlaz, M. A., Jonsson, S., Björn, A., Rowland, S. J., Thompson, R. C., Galloway, T. S. & Yamashita, R. 2009. Transport and release of chemicals from plastics to the environment and to wildlife. *Philosophical Transactions of the Royal Society London. Series B, Biological Sciences,* 364**,** 2027-2045.

Thiel, M. 2011. Chemical Communication in Peracarid Crustaceans. *In:* BREITHAUPT, T. & THIEL, M. (eds.) *Chemical Communication in Crustaceans.* New York, NY: Springer New York.

Thompson, R. C., Olsen, Y., Mitchell, R. P., Davis, A., Rowland, S. J., John, A. W., Mcgonigle, D. & Russell, A. E. 2004. Lost at sea: where is all the plastic? *Science,* 304**,** 838-838.

Ugolini, A. 1996. Jumping and sun compass in sandhoppers: an antipredator interpretation. *Ethology, Ecology and Evolution,* 8**,** 97-106.

Ugolini, A., Pasquali, V., Baroni, D. & Ungherese, G. 2012. Behavioural responses of the supralittoral amphipod Talitrus saltator (Montagu) to trace metals contamination. *Ecotoxicology,* 21**,** 139-147.

Ugolini, A., Ungherese, G., Ciofini, M., Lapucci, A. & Camaiti, M. 2013. Microplastic debris in sandhoppers. *Estuarine, Coastal and Shelf Science,* 129**,** 19-22.

Ungherese, G., Cincinelli, A., Martellini, T. & Ugolini, A. 2012. PBDEs in the supralittoral environment: The sandhopper *Talitrus saltator* (Montagu) as biomonitor? *Chemosphere,* 86**,** 223-227.

Ungherese, G. & Ugolini, A. 2009. Sandhopper solar orientation as a behavioural biomarker of trace metals contamination. *Environmental Pollution,* 157**,** 1360-1364.

Von Moos, N., Burkhardt-Holm, P. & KöHler, A. 2012. Uptake and Effects of Microplastics on Cells and Tissue of the Blue Mussel *Mytilus edulis* L. after an Experimental Exposure. *Environmental science & technology,* 46**,** 11327-11335.

Wegner, A., Besseling, E., Foekema, E., Kamermans, P. & Koelmans, A. 2012. Effects of nanopolystyrene on the feeding behavior of the blue mussel (*Mytilus edulis*). *Environmental Toxicology and Chemistry,* 31**,** 2490-2497.

Weis, J. 2014a. Respiration and Metabolism. *Physiological, Developmental and Behavioral Effects of Marine Pollution.* Springer Netherlands.

Weis, J. S. 2014b. Feeding and Digestion. *Physiological, Developmental and Behavioral Effects of Marine Pollution.* Springer Netherlands.

Wildish, D. J. 1988. Ecology and natural history of aquatic Talitroidea. *Canadian Journal of Zoology,* 66**,** 2340-2359.

Wong, B. B. & Candolin, U. 2015. Behavioral responses to changing environments. *Behavioral Ecology,* 26**,** 665-673.

Wright, S. L., Thompson, R. C. & Galloway, T. S. 2013. The physical impacts of microplastics on marine organisms: a review. *Environmental Pollution,* 178**,** 483-492.

Zala, S. M. & Penn, D. J. 2004. Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. *Animal Behaviour,* 68**,** 649-664.

Chapter 2

Trophic transfer of microplastics does not affect fish personality.

Louise Tosetto, Jane E Williamson, Culum Brown

SAEF lab, Department of Biological Sciences, Macquarie University, North Ryde, Sydney NSW 2109

Corresponding author: Louise Tosetto

***Formatted for Journal: Current Biology**

SUMMARY

Microplastics are ubiquitous in the marine environment and pose physical and chemical risks to marine organisms (Law and Thompson, 2014). Their small size makes microplastics bioavailable to a range of organisms and studies to date have reported ingestion across the food chain (Thompson et al., 2004). While a few studies have demonstrated physical transfer of microplastics between organisms (Eriksson and Burton, 2003, Farrell and Nelson, 2013, Setälä et al., 2014), whether contaminants can be transferred over different trophic levels or what the subsequent ecological impacts of such transfer might be remain unknown. Microplastics accumulate on shorelines (Browne et al., 2011), thus coastal-intertidal areas were used as a model system to assess if the contaminants associated with microplastics could move through the food chain. We exposed beachhoppers (*Platorchestia smithi*) to environmentally relevant concentrations of microplastics and then fed them to Krefft's Frillgobies (*Bathygobius krefftii)*, teleost fish that inhabit shallow coastal ecosystems. We assessed personality in the fish to see if any changes could be attributed to trophic transfer of microplastics, as even subtle changes in behaviour can have a large effect on the wider ecosystem. Exploring behavioural alterations in response to contaminant exposure is a developing area in ecotoxicology due to its sensitivity (Oulton et al., 2014). While gobies readily ingested contaminated beachhoppers, we detected no effect of microplastic trophic transfer on fish personality relative to control groups. Fish from both treatments became shyer over time possibly due to familiarisation in behavioural assays (Kieffer and Colgan, 1992, Brown, 2001). Studies assessing a suite of behaviours with longer-term exposure to microplastics are required to determine if microplastics are providing additional exposure pathways for pollutants in the food web.

HIGHLIGHTS

- Novel study assessing trophic transfer of microplastics and associated contaminants
- Fish readily consumed prey contaminated with microplastics
- Trophic transfer of microplastics did not affect fish personality

RESULTS

This study is the first to examine changes in fish behaviour resulting from the trophic transfer of microplastics and associated contaminants via prey. Trophic interactions provide a passage for contaminants and pollutants to move through the food web (Borgå et al., 2004) and many of the toxins associated with plastics can alter behaviour (Söffker and Tyler, 2012), therefore, this study focussed on the consequences of contaminant transfer rather than physical transfer of microplastics. Hormone levels can influence boldness in fish (Chang et al., 2012, King et al., 2013), and contaminants can alter endocrine systems, with many having a feminizing effect (Vos et al., 2000). Therefore we predicted a shift in the personality of affected fish from bold to shy. Personality - individual differences in behaviour maintained through time and across contexts - has both ecological and evolutionary consequences (Réale et al., 2007, Sih et al., 2012). Variation in personality traits such as activity or boldness can affect food acquisition and social encounters (Stamps, 2007) with bolder individuals benefiting in a foraging and social context (Smith et al., 2009). A shift in a population from bold to shy may have consequences for the local community, thus changes to personality on an individual level may be an indicator of wider ecosystem effects (Weis et al., 2001). Behavioural responses provide useful markers of pollution effects on individuals, potentially performing as reliable and economical indicators of sublethal affects of pollutants (Weis, 2014). Accordingly, there is growing emphasis in ecotoxicology on examining changes in behaviour in response to exposure to contaminants, which is far more sensitive than the standard LD50 (median lethal dose) approach (Oulton et al., 2014).

Trophic transfer of microplastics and contaminants via beachhopper predation

To establish if microplastics provided an additional exposure pathway for contaminants to move through the food web, *B. krefftii* were fed contaminated beachhoppers (treatment) or uncontaminated beachhoppers (control) and behavioural assays were used to assess changes in personality. Beachhoppers are primary consumers that inhabit the sediment in the supralittoral zone of the beach and gobies inhabit the shallows of coastal ecosystems including amphipods in their diet (Souza et al., 2014). Gobies are intermediate consumers forming an important ecological link between invertebrates and larger predatory fish (Leitão et al., 2006) making them an ideal species to assess trophic transfer. Beachhoppers in the treatment group were exposed to beach sand containing 3.8% microplastics and those in the control group were

exposed to sand with no microplastics for 72 hours. Fish behaviour was tested using combined emergence (Brown and Braithwaite, 2004) and open field trial (Archard and Braithwaite, 2011) tests. These behavioural assays quantify a range of boldness and exploration variables that are used as proxies for overall personality in individual fish. Ten boldness variables were recorded for each trial, including emergence time, total time in the safety of the start box, time spent exploring the more exposed parts of the arena and overall activity level (See Figure 5 and Table 2 for full description of variables tested). Three trials were carried out before fish were fed beachhoppers and a further three trials were carried out post exposure, with at least seven days between each trial. To reduce familiarity in the behavioural tests, trials two and five were set up with a green turf background rather than the sandy shade cloth that was used in the other four trials (Figure 5).

Behavioural consequences of contaminated prey ingestion

The ten behavioural variables measured were highly correlated (Spearman's rho, $p<0.01$), thus Principle Component Analysis (PCA) was used to condense variables. PCA condensed the variables to just two components that accounted for 56% of the total variance. Component score coefficients showed that boldness variables such as emergence time, total start box time and time spent in more risky parts of the arena, were the largest contributors to PC1 while exploratory variables such as time spent around the cover and the more exposed and potentially dangerous areas of the arena contributed most to PC2 (Table 1).

Table 1. PCA loadings for each of the behaviour variables in the personality tests. The corresponding code for each behaviour is also provided. Variables in bold contribute the highest amounts to each. See Figure 5 and Table 2 for detailed descriptions of behaviours tested

Behaviour	Code	Boldness (PC1)	Exploration (PC2)
Full Emergence	FE	**-0.372**	0.188
Start Box	SB	**-0.423**	0.161
Cover	CV	0.280	**-0.514**
Bottom Row	BR	0.246	**0.513**
Middle Row	MR	**0.379**	0.260
Top Row	TR	**0.365**	-0.146
Grids adjacent to Start Box	G0	0.213	-0.119
Centre Grid	G5	0.285	0.091
Bottom Centre Grid	G8	0.287	**0.523**
Activity	AC	0.241	-0.164
Eigen Value		4.99	1.44
Proportion variance Explained		49.4%	6.4%

Repeatability was assessed across three trials before and three trials after fish were fed beachhoppers for both PCs. All trials were repeatable for PC1 (Spearman's Rho $p<0.05$) suggesting that the personality traits were robust. Only trials five and six were repeatable for PC2 ($p <0.01$). When repeatability was assessed for treatment and control groups separately, there was no difference in repeatability for either PC1 or PC2 before or after exposure to treatments. This suggests that consumption of plastic contaminated prey items did not affect repeatability of behavioural traits in *B. Kreftii*.

Exposure to a microplastic contaminated diet did not affect boldness or exploration in fish. No significant interactions occurred between exposure to beachhoppers (before and after) and treatment (contaminated or not) for either PC1 (boldness; Two-factor ANOVA, $F = 0.04$, $p=0.84$) or PC2 (exploration; $F = 0.28$, $p = 0.60$). There was a significant effect of exposure to hoppers on the PC1 scores ($F = 18.62$, $p < 0.001$) with fish in both treatment and control groups decreasing in boldness following exposure to the beachhopper diet. In scores indicating exploratory behaviour (PC2), there was a significant effect of treatment ($F=6.85$, $p < 0.01$), with the control groups showing greater exploratory tendencies both before and after exposure (Figure 1).

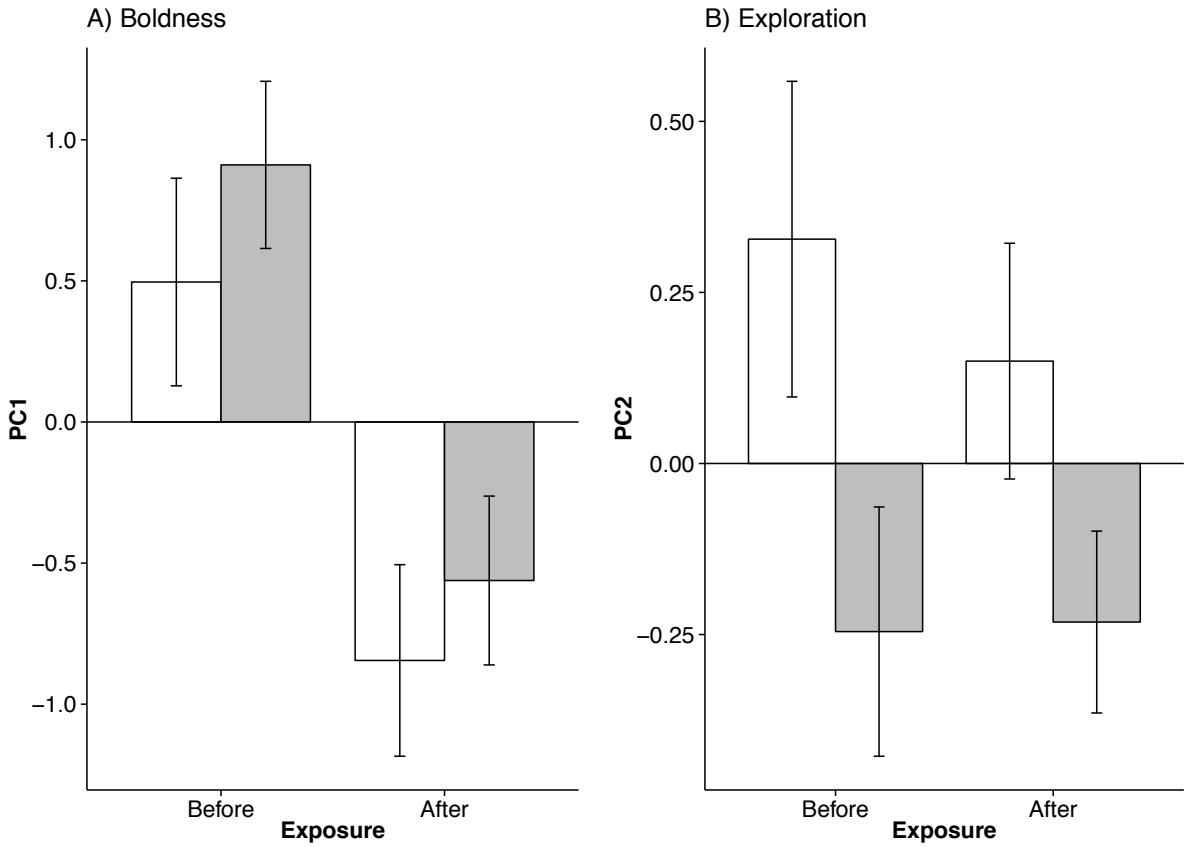

Figure 1. Effect of microplastic exposure on principal component coefficient scores of Krefts gobies monitored in behavioural trials. Control groups are indicated by white bars, treatment groups are indicated by grey bars. Data are shown as means ±SE. A) Behaviour indicating boldness decreased significantly for both treatment groups following exposure. B) Fish in the control group were far more exploratory than those in the treatment group, however any changes following exposure were not significant. (n=14 fish per treatment).

Similarly, boldness and exploration did not significantly differ across trials for either treatment; PC1 ($F = 0.83$, $p = 0.53$) or PC2 ($F = 0.28$, $p = 0.60$). There was a significant effect of trial on boldness ($F= 4.00$, $p< 0.001$), Post-hoc comparisons using the Tukey's HSD test showed that scores in trial six were significantly less ($P < 0.05$) than those in trials one and two. This suggests that boldness in fish decreased over trials. Scores indicative of exploratory behaviour were also significantly affected by trial ($F = 2.38$, $p<0.05$). Tukey's HSD tests showed that exploration scores in trial two were significantly lower to trials one and three at the 0.05 level of significance. The different arena in trial two may have influenced the increased time exploration in the more exposed areas of the arena. No corresponding change in trial five occurred, suggesting that after five trials fish had habituated to the behavioural assay. An effect of treatment was observed in PC2, with fish in the control group scoring higher than those in the treatment group across all trials ($F=67.17$ $P <0.01$) (Figure 2).

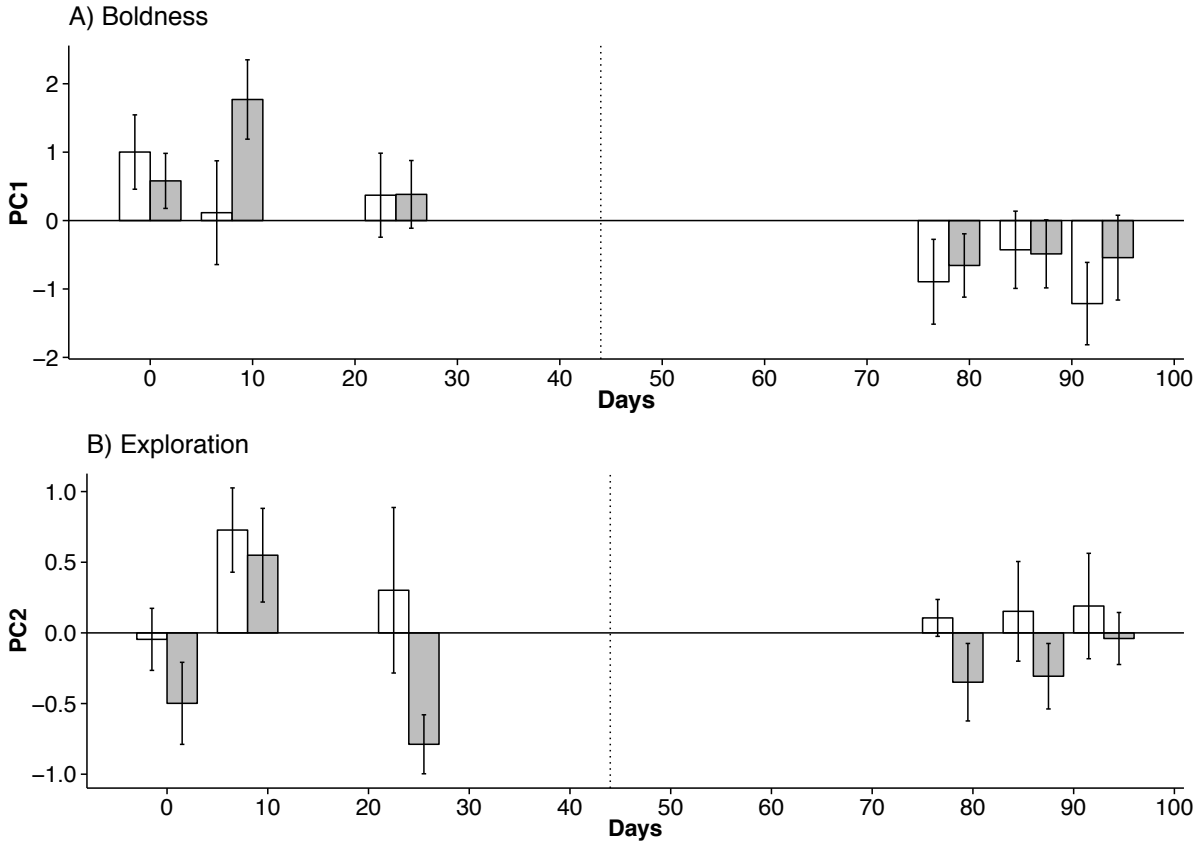

Figure 2. Effect of trial on principal component coefficient scores of Krefts gobies monitored in boldness – exploration trials. Fish in the control group (indicated by the white bars) were more exploratory than those in the treatment group (indicated by grey bars) across all trials. The dotted line indicates the start of plastic exposure. Data are shown as means ± SE. A) Scores indicative of boldness decreased over the six trials as fish became increasingly shy. B) (n = 14 fish per treatment).

There were some key variables contributing to the PCs that were notable. Independent analyses of variables indicative of boldness, such as emergence time and total time in the start box, showed that over the experiment, fish took significantly longer to emerge and spent a larger amount of time in the start box. Increasing emergence and start box time corresponded with a decrease in time spent in the risky section of the arena. Exploratory behaviour was more varied. Fish spent significantly less time exploring the exposed areas of the arena and decreased in activity levels over time. Exploration around the cover of the start box, that provided fish with a sheltered environment to explore while remaining safe, was the only variable that did not change significantly over the experiment (Figure 3).

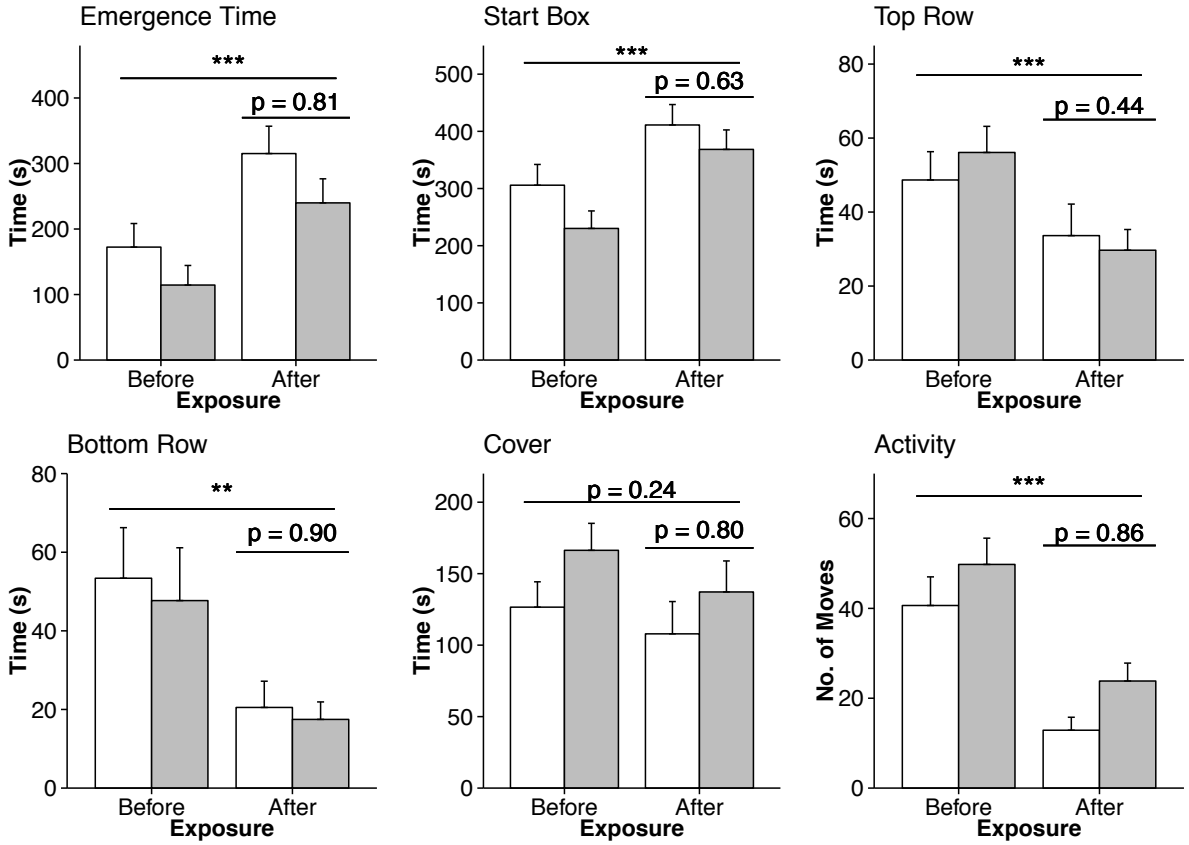

Figure 3. Effects of plastic exposure on control (white bars) and treatment (grey bars) groups for individual variables. Data are shown as means ± SE. Asterisks indicate significant difference ($p < 0.05$). A-C: Key variables indicative of boldness. A) Time for the fish to emerge in full. B) The total time fish spent in the start box. C) The total time fish spent in the top row of the arena. D-F: Key exploratory variables. D) Time spent in the bottom, or more exposed, area of the arena. E) Time spent exploring around the cover of the start box. F) The activity levels of the fish. There was no significant interaction between exposure and treatment for any variable indicated by p values as indicated above. All variables were significantly different before and after exposure except the time spent under cover of the start box. (n = 14 fish per treatment).

DISCUSSION

This is the first study to assess possible behavioural consequences of trophic transfer of microplastics and associated contaminants; in this case through consumption of contaminated prey by a fish. Behavioural responses to contaminants are far more sensitive indicators than the standard LD50 methodology given they can be used to detect subtle sublethal effects of pollutants (Oulton et al., 2014). Moreover, changes in the behaviour of individual species can have wide-ranging effects on ecosystem function (Wong and Candolin, 2015). Given that toxic contaminants found in plastics have been associated with endocrine disruption (Söffker and Tyler, 2012), we expected a shift in behaviour of affected fish from bold to shy. Here we show that, contrary to expectations, transfer of microplastics from beachhoppers to fish did not affect fish personality. There was, however, an overall shift in behaviour with fish becoming shyer over the course of the experiment irrespective of treatment, perhaps due to fish habituating to the experimental tasks. Exploratory behaviour remained constant throughout the experiment, possibly due to the variation in individuals between treatments, with control fish being more exploratory throughout all trials. Gobies were randomly assigned to treatments and there was no difference in sizes of individuals between the groups.

Concern surrounding microplastics is largely due to the associated contaminants and their capacity to bioconcentrate through the food web (Teuten et al., 2009). Whether microplastics are a viable pathway for contaminant exposure is currently under debate. Conceptual models assessing the rates of desorption in organisms suggest that microplastics do not provide a relevant exposure pathway (Gouin, 2011, Koelmans, 2013, Koelmans, 2014). However, positive relationships between microplastic consumption and increased contamination concentration in animal tissue have been demonstrated in seabirds (Ryan et al., 1988), amphipods (Chua et al., 2014) and bivalves (Avio et al., 2015). Furthermore, recent studies suggest that desorption rates of contaminants in gut surfactant are 30 times faster than in seawater for some contaminants, with pH and body temperature also influencing the rate (Bakir et al., 2014). Polyethylene microplastics deployed in the marine environment have the capacity to absorb contaminants (Tosetto et al. 2016, in review). These contaminated microplastics can accumulate in beachhoppers following consumption, and internal plastics can increase the contaminant load of the organism (Tosetto et al. 2016, in

review). Given current understanding of microplastics and contaminants we expected to see a change in fish personality following consumption of contaminated beachhoppers.

The lack of impact of microplastic contamination on goby personality may be due to the short study duration, background contamination levels, or to a more complex association with plastics and pollutants. When directly exposed to marine contaminated microplastics, Japanese meduka (*Oryzias latipes)* suffered hepatic distress but not until two months post initial exposure, suggesting that a certain contamination threshold may be needed before a physiological effect is observed (Rochman et al., 2013). It is possible that the short duration of this study may not have been long enough to reach the contamination threshold for either *P. smithi* or *B. Kreftii*. Given that the life span of these animals is up to eight months and several years respectively, it is possible they are exposed to far greater amounts of plastics and associated pollutants in their lifetime. Furthermore, polymer nature, weathering intensity and pollutant type can all influence how contaminants interact with plastic and organisms (Mato et al., 2001, Frias et al., 2010) highlighting that relationships between microplastics, contaminants and organisms can be complex. Additionally, theoretical models predict that the consumption of microplastics does not increase the burden of pollutants in fish given the background levels already present in the environment (Gouin, 2011). Given the fish used in this study were collected from an urbanised area in Sydney, Australia and fed a diet of commercial frozen food during acclimation, it is plausible that the experimental effects of plastic consumption were not discernable from the background levels in our wild caught fish.

Using personality to assess anthropogenic impacts on individuals is a relatively new area of research. Personality is a major driver in population dynamics that can influence competitive interactions and how animals deal with changes in their environment (Montiglio and Royauté, 2014), particularly those attributed to anthropogenic impacts such as environmental contamination (Wong and Candolin, 2015). Assessing how contaminants alter personality traits provides a far more subtle way of establishing effects in comparison to the standard LD50 approach commonly used in ecotoxicology (Oulton et al., 2014). Recent studies have examined how direct exposure to pollutants can change fish personality. A study assessing responses of Siamese fighting fish (*Betta splendens*) to exposure of the hormone 17 α-ethinylestradiol reported a decrease in overall activity, lowered repeatability and weakened correlations of affected fish,

however, there were no trial and treatment interaction effects across the boldness assays (Dzieweczynski et al., 2014). Following long term increased CO_2 exposure, simulating scenarios of ocean acidification, female sticklebacks (*Gasterosteus aculeatus)* showed changes to lateralisation and exploration, however, fish exhibited familiarisation with the boldness task in the later stages of the experiment (Jutfelt et al., 2013). Moreover, in studies assessing effects of the pharmaceutical oxazepam on European perch (*Perca fluviatilis*), there was evidence of increased activity in the treatment group but again, no effect on boldness in either the control or treatment groups (Brodin et al., 2013, Brodin et al., 2014).

While our study did not see a change in exploration, activity or repeatability of behaviour in the fish in the treatment group, it did have comparative findings for boldness suggesting that habituation or familiarisation in boldness assays is a factor that should be taken into account when assessing personality. In multiple trials, Kieffer and Colgan (1992) have demonstrated that the longer fish are exposed to a similar task the more inflexible behaviour comes, with less motivation to emerge and subsequently less time given to exploring an area. Moreover, fish will decrease in activity as they become familiar with a tank environment (Brown, 2001). It is key to note that while our fish became familiar with the test environment, there was no difference in habituation, or familiarization across the treatment and control group that could indicate an effect by our treatments to these processes.

Overall, this study found that short term trophic transfer of microplastics via prey does not affect the personality of fish. Similar to previous studies assessing how anthropogenic influence affects behaviour, there was familiarisation of the behavioural tests over time. Challenges of animals learning and habituating to an experiment will persist across any longitudinal experiments. There are difficulties in obtaining an accurate picture of the impacts of microplastics due to their pervasiveness across the marine environment and studies using organisms raised in the laboratory may be the only way to make an accurate initial assessment to help understand the mechanics of the process. Furthermore, taking an all-inclusive approach to behavioural experiments that assess the impacts of microplastics on behaviours applicable to foraging, sexual activity, sociality and learning are required to understand the broad impacts of plastic exposure. Future studies should set up longer-term exposure experiments and preferably intergenerational exposure studies that assess a suite of personalities and

behaviours to get a more comprehensive picture of changes in behaviour in response to microplastics.

METHODS

Ethics Statement

Experimental methods in this study conformed to the standards set by Macquarie University Animal Ethics committee (ARA 2014 / 003-7). Fish collections were conducted under NSW Fisheries Scientific Collection Permit number P08/0010-4.2.

Study Animal

Krefft's frillgoby (*Bathygobius krefftii*) are small, ray finned marine fish. They grow to a maximum length of 9cm and inhabit shallow seagrass beds and rocky estuaries (Kuiter, 1993). The fish were caught, using hand nets from Chowder Bay, an urbanised area in Sydney, Australia (33°50'24"/151°15'13"). A total of 33 fish were collected and tested for behaviour. Fish were acclimated in the laboratory for two months prior to testing. Individuals were housed in groups of eight in four flow-through seawater aquaria (64 cm x 42 cm x 26 cm: 70L), held in the seawater facility at the Sydney Institute of Marine Science (SIMS), Australia. All aquaria were maintained at the same seawater flow rate (1L / 1min-1), temperature (16-22°C), with a gravel substrate and aeration from an air stone. Fish were fed live black worms (*Lumbriculus variegates*) during the one week settling period and then a mixture of frozen commercial 'Hikari Bio-Pure' Mysis shrimp, Brine Shrimp and Black Worms. Tanks were cleaned weekly, and physically enriched with terracotta pots. Lighting came from overhead fluorescent tubes for 12 hours per day. Four weeks before testing, fish were lightly anesthetized using a solution of 50 mg / 1 MS222 buffered with sodium bicarbonate (fish placed in a bucket containing 1.5 L solution until subdued), their total length was measured and fish were tagged using Visible Implant Fluorescent Elastomer tags (VIE: Marine Technology, Inc 2008) to assist individual identification. Fish recover from tagging almost immediately and it has no long-term effects on behaviour (White and Brown, 2013).

Boldness-Exploration Assays

Two different arenas were set up for the boldness-exploration tests. Both were established in 70 L aquariums identical in size to the housing tanks. (1) The bottom of the aquarium was covered with 5cm of sand from a nearby beach and the sides lined with sand colour shade cloth (Coolaroo Sandstone 70% UV). (2) Shell grit (3 cm) covered the bottom of the tank and the sides lined with green turf (Tuff Turf Natural Synthetic). The aquarium was filled with 150 mm water and a dark plastic box (140 mm

x 140 mm x 190 mm high) was placed at one end of the arena. An air stone was placed behind the start box for aeration. Fish were tested individually in both arenas. After capture with a hand net from the housing tank, the test fish was placed into the start box, and the lid was placed on top. The fish was left for a two minute settling period after which a remote pulley lifted the door, allowing the individual to emerge from a door (30mm x 60 mm) and explore the test arena. To eliminate impacts of human observers on fish behaviour, fish were observed remotely via a computer monitor connected by firewire (Belkin FireWire cable IEEE 1394) to a digital camera mounted over the test arena. At the end of the test period (600 s) fish were netted and returned to their housing tank.

Experimental Treatments

Six individuals were excluded from analyses due to mortality; 28 fish remained for analysis of personality. All fish were put through behavioural trials before exposure to beachhopper treatments. The test was repeated three times prior to treatment, trials one and three were in arena 1 (sandy) and the second trial in arena 2 (turf). There was at least a 7 day interval between each trial. Following initial tests, gobies were randomly assigned to two treatment groups and housed accordingly. There were no significant differences between sizes of individuals between treatment and control once randomly assigned ($F = 0.265$, $p = 0.61$). Treatment fish (n=14) were fed plastic contaminated beachhoppers that had been exposed to 25 g sediment with 1 g microplastics (3.8% Dry Weight), for 72 hours, while control fish (n=14) were fed beachhoppers that had been exposed to 26 g clean sediment only, for 72 hours. Fish were fed two to three beach-hoppers every two days for four weeks. The second round of testing commenced at the end of the first week of the beachhopper diet. Three trials were undertaken after plastic exposure. Trials four and six were once again undertaken in arena 1 while trial five was carried out in arena 2. There was at least seven days between trials four, five and six (Figure 4).

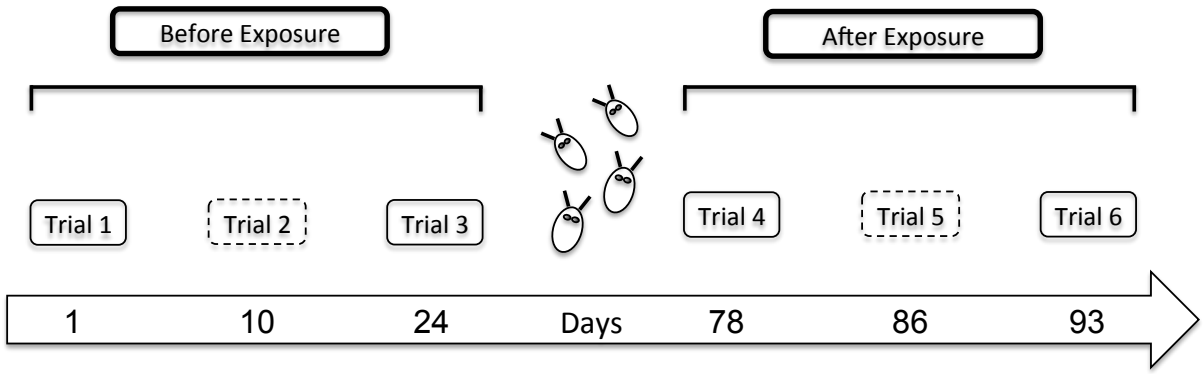

Figure 4. Schematic diagram of behavioural trials. Beachhoppers indicate where diet changed from commercial fish food to beachhoppers. Trials with solid lines indicate arena 1 (Sand) and dotted lines indicate arena 2 (Turf).

Scoring Behaviour

Videos were scored for emergence, exploration and activity levels using Etholog TM (Ottoni, 2000). Emergence time was recorded when the caudal fin of the fish was observed out of the start box. Fish that failed to emerge were given the maximum score of 600s. To quantify exploration, i.e., the amount of time fish spent in various parts of the arena, a grid was placed over the tank that divided the arena into ten sections (Figure 5). The various behaviours scored are outlined in table 2. The start box and cover were considered safe areas for the fish, the top and middle rows were identified as risky, and the bottom row and centre was deemed dangerous as they were furthest from any cover. The total time spent in each grid was recorded, a fish was not deemed to be in a new grid until the caudal fin had moved into the area. The number of movements made by the fish over the test period was a proxy for activity.

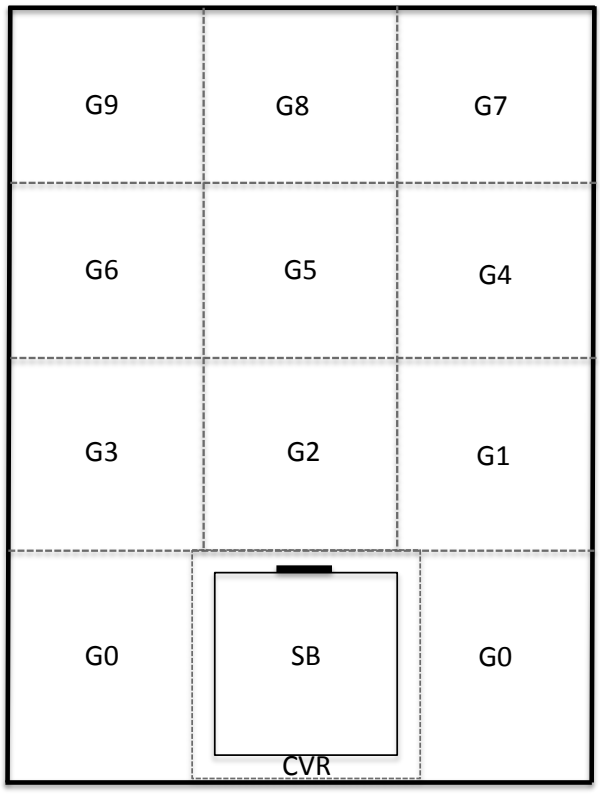

Figure 5. The test arena used for scoring fish boldness and exploration.

Statistical Analysis

Table 2. The ten variables used in the fish personality testing.

Code	Variable	Behaviour	Description
FE	Full Emerge	Boldness	The time for fish to emerge in full from the start box, recorded when the caudal fin was out of the box.
SB	Start Box	Boldness	The total time spent in the start box. The start box was a safe area and fish would return to the start box after emergence. The total time (initial time + return time) is indicated here
TR	Top Row	Boldness	Total time fish spent in grids 1, 2 and 3.
MR	Middle Row	Boldness	Total time fish spent in grids 4, 5 and 6.
G5	Grid 5	Boldness	Although related to MR, the centre of the arena was deemed one of the most dangerous areas for the fish to move into.
CV	Cover	Exploration	The total time spent exploring the area around the start box. Cover was defined as 2 cm around the start box.
BR	Bottom Row	Exploration	Total time fish spent exploring the bottom of the arena, a combination of time spent in grids 7, 8 and 9.
G0	Grid 0	Exploration	Total time spent the top area of the arena once outside of the cover of the start box
G8	Grid 8	Exploratory	Related to BR but like G5, a little more risky given it was in the centre of the arena
AC	Activity	Exploratory	The total movements made by the fish over the course of the test

Comparisons of individual variables were made using nonparametric Spearman's rank correlation tests in R Studio. Correlations were made to assess individual consistency in related measures of behaviour. To test for the presence of personality, incorporating boldness and exploration, individual traits were collapsed into principal component scores using Principal Components Analysis (PCA). PCA was undertaken using princomp function in R (R Core Team, 2013). Eigenvectors with a value greater than one were used to describe the variance in the dataset. The principal components were used in future analyses. Repeatability between trials was assessed for the PCs using Spearman's rank correlation test. Two-factor ANOVAs were used to assess significant interactions between exposure and treatment, and between trial and treatment. Where no significant interaction was observed, the interaction effect was dropped and the model run using the lm command in R (R Core Team, 2013). The importance of treatment, exposure and trial were assessed using the drop command.

ACKNOWLEDGEMENTS

This research was funded by the Department of Biological Sciences at Macquarie University. This paper is contribution #xxxx from Sydney Institute of Marine Science.

REFERENCES

Archard, G. A. & Braithwaite, V. A. 2011. Variation in aggressive behaviour in the poeciliid fish *Brachyrhaphis episcopi*: population and sex differences. *Behavioural Processes,* 86**,** 52-57.

Avio, C. G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., D'errico, G., Pauletto, M., Bargelloni, L. & Regoli, F. 2015. Pollutants bioavailability and toxicological risk from microplastics to marine mussels. *Environmental Pollution,* 198**,** 211-222. doi: 10.1016/j.envpol.2014.12.021

Bakir, A., Rowland, S. J. & Thompson, R. C. 2014. Enhanced desorption of persistent organic pollutants from microplastics under simulated physiological conditions. *Environmental Pollution,* 185**,** 16-23. doi: 10.1016/j.envpol.2013.10.007

Borgå, K., Fisk, A. T., Hoekstra, P. F. & Muir, D. C. 2004. Biological and chemical factors of importance in the bioaccumulation and trophic transfer of persistent organochlorine contaminants in arctic marine food webs. *Environmental Toxicology and Chemistry,* 23**,** 2367-2385.

Brodin, T., Fick, J., Jonsson, M. & Klaminder, J. 2013. Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. *Science,* 339**,** 814-815.

Brodin, T., Piovano, S., Fick, J., Klaminder, J., Heynen, M. & Jonsson, M. 2014. Ecological effects of pharmaceuticals in aquatic systems—impacts through behavioural alterations. *Philosophical Transactions of the Royal Society London. Series B, Biological Sciences,* 369**,** 20130580.

Brown, C. 2001. Familiarity with the test environment improves escape responses in the crimson spotted rainbowfish, *Melanotaenia duboulayi*. *Animal Cognition,* 4**,** 109-113.

Brown, C. & Braithwaite, V. A. 2004. Size matters: a test of boldness in eight populations of the poeciliid *Brachyraphis episcopi. Animal Behaviour,* 68**,** 1325-1329.

Browne, M. A., Crump, P., Niven, S. J., Teuten, E., Tonkin, A., Galloway, T. & Thompson, R. 2011. Accumulation of microplastic on shorelines woldwide: sources and sinks. *Environmental Science & Technology,* 45**,** 9175-9179. doi: 10.1021/es201811s

Chang, C., Li, C.-Y., Earley, R. L. & Hsu, Y. 2012. Aggression and related behavioral traits: the impact of winning and losing and the role of hormones. *Integrative and Comparative Biology,* 52**,** 801-813.

Chua, E., Shimeta, J., Nugegoda, D., Morrison, P. D. & Clarke, B. O. 2014. Assimilation of Polybrominated Diphenyl Ethers from microplastics by the marine amphipod, *Allorchestes Compressa. Environmental Science & Technology,* 48**,** 8127-8134. doi: 10.1021/es405717z

Dzieweczynski, T. L., Campbell, B. A., Marks, J. M. & Logan, B. 2014. Acute exposure to 17α-ethinylestradiol alters boldness behavioral syndrome in female Siamese fighting fish. *Hormones and Behavior,* 66**,** 577-584.

Eriksson, C. & Burton, H. 2003. Origins and biological accumulation of small plastic particles in fur seals from Macquarie Island. *AMBIO: A Journal of the Human Environment,* 32**,** 380-384. doi: 10.1579/0044-7447-32.6.380

Farrell, P. & Nelson, K. 2013. Trophic level transfer of microplastic: *Mytilus edulis* to *Carcinus maenas. . Environmental Pollution,* 177**,** 1-3. http://dx.doi.org/10.1016/j.envpol.2013.01.046

Frias, J., Sobral, P. & Ferreira, A. 2010. Organic pollutants in microplastics from two beaches of the Portuguese coast. *Marine Pollution Bulletin,* 60**,** 1988-1992. doi: 10.1016/j.marpolbul.2010.07.030

Gouin, T. 2011. A thermodynamic approach for assessing the environmental exposure of chemicals absorbed to microplastic. *Environmental Science & Technology,* 45**,** 1466-1472. doi: 10.1021/es1032025

Jutfelt, F., De Souza, K. B., Vuylsteke, A. & Sturve, J. 2013. Behavioural disturbances in a temperate fish exposed to sustained high CO2 levels. *Plos ONE,* 8**,** e65825. doi:10.1371/journal.pone.0065825. .

Kieffer, J. D. & Colgan, P. W. 1992. The role of learning in fish behaviour. *Reviews in Fish Biology and Fisheries,* 2**,** 125-143.

King, A. J., Fürtbauer, I., Mamuneas, D., James, C. & Manica, A. 2013. Sex-differences and temporal consistency in stickleback fish boldness. *PLoS ONE,* 8**,** e81116. doi:10.1371/journal.pone.0081116.

Koelmans, A. A. 2013. Plastic as a Carrier of POPs to Aquatic Organisms: A Model Analysis. *Environmental Science & Technology,* 47**,** 7812-7820. doi: 10.1021/es401169n

Koelmans, A. A. 2014. Leaching of plastic additives to marine organisms. *Environmental Pollution,* 187**,** 49-54. doi: 10.1016/j.envpol.2013.12.013

Kuiter, R. H. 1993. *Coastal fishes of south-eastern Australia,* Bathurst, NSW, Crawford House Press.

Law, K. L. & Thompson, R. C. 2014. Microplastics in the seas. *Science,* 345**,** 2. doi: 10.1126/science.1254065

Leitão, R., Martinho, F., Neto, J., Cabral, H., Marques, J. & Pardal, M. 2006. Feeding ecology, population structure and distribution of *Pomatoschistus microps* (Krøyer, 1838) and *Pomatoschistus minutus* (Pallas, 1770) in a temperate estuary, Portugal. *Estuarine, Coastal and Shelf Science,* 66**,** 231-239.

Mato, Y., Isobe, T., Takada, H., Kanehiro, H., Ohtake, C. & Kaminuma, T. 2001. Plastic resin pellets as a transport medium for toxic chemicals in the marine environment. *Environmental Science & Technology,* 35**,** 318-324.

Montiglio, P.-O. & Royauté, R. 2014. Contaminants as a neglected source of behavioural variation. *Animal Behaviour,* 88**,** 29-35.

Ottoni, E. B. 2000. Etholog 2.2 - A tool for the transcription and timing of behavior observation sessions. *Behaviour Research Methods, Instruments & Computers,* 32**,** 4.

Oulton, L. J., Taylor, M. P., Hose, G. C. & Brown, C. 2014. Sublethal toxicity of untreated and treated stormwater Zn concentrations on the foraging behaviour of *Paratya australiensis* (Decapoda: Atyidae). *Ecotoxicology,* 23**,** 1022-1029. doi: 10.1007/s10646-014-1246-2

R Core Team, 2013, R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria

Réale, D., Reader, S. M., Sol, D., Mcdougall, P. T. & Dingemanse, N. J. 2007. Integrating animal temperament within ecology and evolution. *Biological Reviews of the Cambridge Philosophical Society,* 82**,** 291-318. 10.1111/j.1469-185X.2007.00010.x

Rochman, C. M., Hoh, E., Kurobe, T. & Teh, S. J. 2013. Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. *Scientific Reports,* 3**,** 3263. doi: 10.1038/srep03263

Ryan, P., Connell, A. & Gardner, B. 1988. Plastic ingestion and PCBs in seabirds: is there a relationship? *Marine Pollution Bulletin,* 19**,** 174-176. doi: 10.1016/0025-326X(88)90674-1

Setälä, O., Fleming-Lehtinen, V. & Lehtiniemi, M. 2014. Ingestion and transfer of microplastics in the planktonic food web. *Environmental Pollution,* 185**,** 77-83.

Sih, A., Cote, J., Evans, M., Fogarty, S. & Pruitt, J. 2012. Ecological implications of behavioural syndromes. *Ecology Letters,* 15**,** 278-289. 10.1111/j.1461-0248.2011.01731.x

Smith, K. L., Miner, J. G., Wiegmann, D. D. & Newman, S. P. 2009. Individual differences in exploratory and antipredator behaviour in juvenile smallmouth bass (Micropterus dolomieu). *Behaviour,* 146**,** 283-294.

Söffker, M. & Tyler, C. R. 2012. Endocrine disrupting chemicals and sexual behaviors in fish-a critical review on effects and possible consequences. *Critical reviews in Toxicology,* 42**,** 653-668.

Souza, A., Dias, E., Campos, J., Marques, J. & Martins, I. 2014. Structure, growth and production of a remarkably abundant population of the common goby, *Pomatoschistus microps* (Actinopterygii: Gobiidae). *Environmental Biology of Fishes,* 97**,** 701-715.

Stamps, J. A. 2007. Growth‐mortality tradeoffs and ‘personality traits’ in animals. *Ecology Letters,* 10**,** 355-363.

Teuten, E. L., Saquing, J. M., Knappe, D. R., Barlaz, M. A., Jonsson, S., Björn, A., Rowland, S. J., Thompson, R. C., Galloway, T. S. & Yamashita, R. 2009. Transport and release of chemicals from plastics to the environment and to wildlife. *Philosophical Transactions of the Royal Society London Series B, Biological Sciences,* 364**,** 2027-2045. doi: 10.1098/rstb.2008.0284

Thompson, R. C., Olsen, Y., Mitchell, R. P., Davis, A., Rowland, S. J., John, A. W., Mcgonigle, D. & Russell, A. E. 2004. Lost at sea: where is all the plastic? *Science,* 304**,** 838-838. doi: 10.1126/science.1094559

Vos, J. G., Dybing, E., Greim, H. A., Ladefoged, O., Lambré, C., Tarazona, J. V., Brandt, I. & Vethaak, A. D. 2000. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. *CRC Critical Reviews in Toxicology,* 30**,** 71-133.

Weis, J. 2014. *Physiological, Developmental and Behavioral Effects of Marine Pollution,* Netherlands, Springer. doi: 10.1007/978-94-007-6949-6

Weis, J. S., Smith, G., Zhou, T., Santiago-Bass, C. & Weis, P. 2001. Effects of Contaminants on Behavior: Biochemical Mechanisms and Ecological Consequences. *Bioscience,* 51**,** 209-217.

White, G. E. & Brown, C. 2013. Site fidelity and homing behaviour in intertidal fishes. *Marine Biology,* 160**,** 1365-1372.

Wong, B. B. & Candolin, U. 2015. Behavioral responses to changing environments. *Behavioral Ecology,* 26**,** 665-673. doi: 10.1093/beheco/aru183

Appendix

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2014/003-8 **Date of Expiry:** 20 February 2016

Full Approval Duration: *21 February 2014 to 20 February 2017 (36 Months)*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator:
A/Prof Culum Brown
Biological Sciences
Macquarie University, NSW 2109
0439 343 341
Culum.Brown@mq.edu.au

Associate Investigators:
Penelope Carbia 0403 082 950
JennaLee Clark 0404 004 882

Researcher/ Research Students
Louise Tosetto 0406 189 992
Evan Byrnes 0432 761 233

In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above, or
Animal Welfare Officer - 9850 7758 / 0439 497 383

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: **Spatial Learning and Memory Retention in Fish**

Purpose: **4 - Research: Human or Animal Biology**

Aims: **To examine how long fish from contrasting environments retain spatial information**

Surgical Procedures category: 3 - Minor Conscious Intervention

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age/Sex	Total	Supplier/Source
23 – Fish	Intertidal gobies	Mature adult / Any	240 +100	Wild
23 – Fish	Port Jackson Sharks	Any		
		TOTAL	340	

Location of research:

Location	*Full street address*
SIMS	Building 19, Chowder Bay Road, Mosman NSW 2088
Biology Sea Water Facility	Building E8C, Macquarie University, North Ryde, 2109
Fauna Park	209 Culloden Rd, North Ryde, 2109

Amendments approved by the AEC since initial approval:

1. *Amendment #1 - Change transport and capture methods (Exec approved 8 May 2014, ratified by AEC 15 May 2014).*
2. *Amendment #2 – Add a new species - Port Jackson Sharks (Approved at AEC meeting14 August 2014).*
3. *Amendment #3 – Addition of Evan Brynes as a Masters Student(Executive approved, ratified by AEC 11 December 2014)*
4. *Amendment # 4 – Addition of JennaLee Clark as an Associate Investigator(Executive approved, ratified by AEC 11 December 2014)*
5. *Amendment #5 – Addition of 100 Port Jackson Eggs(Executive approved, ratified by AEC 11 December 2014)*
6. *Amendment #6 – Addition of Louise Torsetto as Researcher(Executive approved, ratified by AEC 11 December 2014)*
7. *Amendment #7 – Amending the way to feeding the gobies(AEC approved 19 February 2015)*

Conditions of Approval: N/A

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

Dr Karolyn White (Acting Chair, Animal Ethics Committee) **Approval Date: 19 February 2015**