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Decreased growth and survival in small juvenile fish, after chronic exposure to environmentally relevant concentrations of microplastic☆

Trishan Naidoo*, David Glassom

School of Life Sciences, University of KwaZulu-Natal Westville Campus, Biology Building on University Road, Private Bag X54001, Durban 4000, South Africa

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ABSTRACT

Glassfish, *Ambassis dussumieri* (Cuvier, 1828), was used as a sentinel species to investigate the effects of the ingestion of environmentally relevant microplastic concentrations on juvenile fish growth and survival. Both virgin plastic and plastic collected from an urban harbour were fed to small juvenile fish daily for 95 days. Fish standard length, body depth and mass were recorded at intervals of 20 days, while survival was continuously recorded. All fish were fed tropical flakes, measured at 1.7% of the body mass per tank. Overall, fish in in plastic treatments grew less in body length and body depth compared to those control treatments. Fish mass was also lower in the virgin plastic treatment than control fish; however, the growth in mass was not significantly lower than fish in the harbour plastic treatment. The survival probability of fish in both plastic fed treatments was also lower than fish in controls.

1. Introduction

The ingestion of microplastics, ≤5 mm in length, by fish has been recorded from the early 1970s (Carpenter et al., 1972), when scientists speculated on its negative influence on fish health (Hoss and Settle, 1990). Recently, fish have been documented ingesting microplastics in a variety of water bodies including rivers (Sanchez et al., 2014), shallow coastal estuarine systems (Naidoo et al., 2016), the ocean surface (Choy and Drazen, 2013) and even the deep ocean (Anastasopoulou et al., 2013). Plastic ingestion has been found in both demersal and pelagic feeding guilds (Lusher et al., 2013). Field evidence of any negative effects of this ingestion, for example gut lesions or tissue damage, is challenging to observe and may be limited because of destructive plastic isolation methods, such as acid digestion, or separating any observed effects from other field contaminants (Steer et al., 2017). Manipulative feeding experiments are therefore used to determine the biological (Rochman et al., 2013; Pedà et al., 2016) and ecosystem effects (Bergami et al., 2016) of microplastic ingestion.

Experiments have mainly revealed the negative effects of microplastic ingestion at the tissue, organ and organism levels (Jovanović, 2017). For example, Rochman et al. (2013) showed that discarded low density polyethylene (LDPE) pellets caused changes to the liver tissue of the Japanese medaka, *Oryzias latipes* (Temminck and Schlegel, 1846); and Pedà et al. (2016) observed that polyvinyl chloride (PVC) pellets affected the intestinal structure of the sea bass, *Dicentrarchus labrax* (L. 1758). Such alterations could result in organism changes that include decreased feeding and decreased body mass (Welden and Cowie, 2016). Higher level effects include impaired development and decreased reproductive potential, even by virgin plastic, as shown for the sea urchin *Lytechinus variegate* (Lamarck, 1816) and the oyster *Crassostrea gigas* (Thunberg, 1793) (Nobre et al., 2015; Sussarellu et al., 2016).

Assessing these threats using manipulation experiments, on small juvenile fish is both needed and is ecologically important, as it increases our understanding of the effect that microplastics can have on recruitment (Mazurais et al., 2015). Juvenile fish are already vulnerable to environmental perturbations that can affect their survival at early stages and may be particularly vulnerable to microplastic ingestion (Whitfield, 1990; Lima et al., 2015). They use polluted urban estuaries as nurseries, bringing them in contact with plastic particles at a higher frequency (Lima et al., 2015; Naidoo et al., 2015) and their relative size compared to microplastic particles may make any ingested particles more dangerous or even harder to pass compared to adult fish. Juveniles of commercially important species nursing in such areas could thus affect fisheries in the long term (Markic and Nicol, 2014) especially since there can be a similar number of plastic particles as juvenile fish in estuaries (Lima et al., 2015). Furthermore, it is predicted that the ocean plastic mass will outweigh fish mass by 2050, outlining the necessity to evaluate potential impacts (Jovanović, 2017). Studies on the

☆ Capsule: Chronic exposure to microplastics is shown here to affect the long term growth and survival of juvenile fish.

* Corresponding author.
E-mail addresses: 207513950@stu.ukzn.ac.za (T. Naidoo), glassom@ukzn.ac.za (D. Glassom).

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effects of microplastics on small juvenile fish are scarce and those targeting the chronic long term effects of exposure in an environmentally relevant situation are even more so (Steer et al., 2017). This study has set out to fill this important research gap.

We aimed to assess the long term impact of microplastic ingestion on the growth and survival of juvenile fish. To test this, *Ambassis dussumieri* (Cuvier, 1828) was used as a sentinel species. These glassfish or glassies are common coastal fish that are translucent and cosmopolitan (Anderson and Heemstra, 2003). They are an integral part of the food chain and usually feed in the water column on zooplankton (Forbes and Demetriades, 2008; Dyer et al., 2015). They are thus likely to interact with the high microplastic concentrations found in urban estuaries (Naidoo et al., 2015; Clark et al., 2016). It was hypothesised that that growth and survival would decrease in *A. dussumieri* exposed to environmentally realistic concentrations of microplastic. The objectives were to feed *A. dussumieri* four different plastic types of both virgin plastic and plastic stranded in a polluted harbour and measure their growth and survival over three months.

2. Materials and methods

2.1. Tank setup

In total, 450 juvenile *A. dussumieri* were collected from Durban Harbour (29° 52′S, 31° 04′E), using a fine mesh dip net. The fish had an initial mean standard length and standard deviation of 21.36 ± 4.05 mm. They were tagged and acclimated for a month before being subjected to microplastic treatments. Ten fish were kept per 20 L tank. Nine fish per tank were tagged with a green, red or blue fluorescent elastomere (Northwest Marine Technologies, Inc.), while the 10th fish was identified by the lack of a tag. Tags were inserted at one of three positions with a syringe (see Supplementary Information). Treatments were either fish that were fed virgin plastic, plastic collected from the industrialised Durban Harbour termed 'harbour plastic' or no plastic. These treatments were all replicated three times and maintained in filtered seawater at a salinity of 35, a constant temperature of 25 °C and a 12 day: 12 h night light regime. A set of five tanks connected to a single sump and protein skimmer constituted a single recirculation system. This set of five tanks formed a single replicate for each treatment. The flow rate from each sump was 2500 L/h, which was split and equally distributed using valves between adjacent tanks. A complete water change was done every two weeks, while fish faeces and leftover food particles, including plastic particles, were siphoned out daily after a 30 min feeding event. This study was done under ethical clearance by the animal ethics research committee of the university of KwaZulu − Natal (AREC/011/016D).

2.2. Plastic preparation and feeding

A mixture of virgin plastic types was fed to *A. dussumieri*. These were mixed in the following proportions by mass: 9-parts film material composed of polyethylene (high density): 5-parts polyvinyl chloride fragments: 1-part grinded pellet material composed of polyethylene (high density): 1-part polystyrene. These represented 95% of the plastic types found in water samples from the harbour, in the same proportion by abundance (Naidoo et al., 2015). The same proportions of plastics were used from samples collected directly from the harbour and none were treated or cleaned before use. This was termed the harbour plastic treatment to differentiate them from the virgin plastic treatment. For each plastic type, particles were ground with a coffee bean grinder and only those between 1000 μm and 250 μm in length, which accounted for 73% of plastics size range found in the surface water tows within the harbour, were used in the experiment. Since the glassfish mainly feed on zooplankton within the water column (Dyer et al., 2015), we hypothesised that these proportions would be relevant to the feeding behaviour of these fish.

Studies should use environmentally relevant plastic concentrations of the common types and shapes encountered in situ (Huvet et al., 2016; Lenz et al., 2016; Lusher et al., 2017), as many studies have used concentration of plastics that are generally not encountered in the environment e.g. 1000 particles per mL (Cole and Galloway, 2015). Therefore, the highest concentration of plastic found in Durban Harbour was used in this study (Naidoo et al., 2015). This equated to 1.769 g per 10,000 L of surface water which was 0.051 g per 290 L treatment setup or 0.010 g of plastic per tank as a daily feed at the start of the experiment. During feeding, the pump in each sump was switched off. Fish in each tank were fed 1.7% of their total body weight daily with tropical flake food (Qualipet®). Fish in plastic treatment tanks were given one part of the plastics mixture in addition to every five parts of fish food, by mass. Fish food and ground plastics were sprinkled on the surface of each tank and the fish were allowed to feed ad libitum. As fish numbers decreased through mortality, the food proportions were adjusted accordingly for that tank and therefore overall plastic concentrations in treatments also decreased. After 30 min, faeces and plastic debris were siphoned out and the pumps where switched on again for floating debris to pass through the tank outlet and get collected on a filter. Plastics were clearly visible embedded in the faeces of fish in plastic treatments after an overnight check, indicating that plastics were being consumed and defecated (see Supplementary Information).

2.3. Retention

Since plastic retention data are still scarce for fish and may play a pivotal role in the magnitude of any negative effects (Jovanović, 2017), a pilot study was thus conducted to determine the retention of PVC particles in the glassfish. Five 20 L tanks were used in the experiment and five *A. dussumieri* were kept in each tank. The fish had an average total length of 28.52 ± 2.14 mm and a mass of 0.183 ± 0.042 g. An initial exposure of 0.05 g ground PVC fragments was added to the surface of the water in each tank without food. Each particle weighed approximately 0.001 g. Fish were not fed for the four day exposure. After the 10 min exposure and thereafter on each day, one fish from each tank was euthanised in 99% ethanol and stored and digested whole, following Naidoo et al. (2017), to obtain a mass of the PVC particles that had been consumed. On each day, 95% of the water from each tank was siphoned out and the plastics remaining in the water column were isolated and weighed. At the end of the experiment all water was removed to and checked for any remaining PVC particles. Although only the PVC polymer was used in this pilot experiment, as opposed to the rest of the study, it served to indicate how long *A. dussumieri* could retain particles after the initial feed (Fig. 1).

2.4. Determining growth, condition and survival

The Standard Length (SL) and Body Depth (BD) of each fish was measured to the nearest 0.1 mm with a pair of calipers at time intervals of 19 (n = 412), 38 (n = 288), 68 (n = 192) and 92 days (n = 82). Their mass (g) was also recorded with a mass balance to the nearest 0.001 g. Any dead fish were taken out, daily recorded for survival data and stored in 10% formalin for analysis of their gut plastic content. This was done following Naidoo et al. (2017), to isolate and enumerate consumed plastic particles. Two fish from each tank were also culled before measurements were taken, at each time interval, to determine if plastics accumulated in them as the experiment progressed. This was changed to one fish if fewer fish were present in the tank as time progressed, to even out densities, as different stocking densities could affect the water quality that the fish experience. Culled fish were stored in 10% formalin and also digested to determine their plastic content.

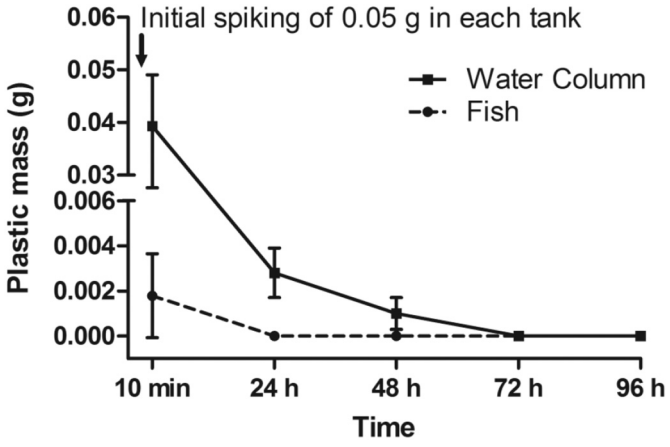

Fig. 1. Particle retention time of PVC in the gut of *Ambassis dussumieri*, from an initial dose of 0.05 g. Bars represent the standard deviation of the mean. 1.5 – column fitting image.

2.5. Comparing length, body depth and mass

The fish that survived throughout the experiment were used to compare fish growth among treatments. Initially boxplots were created in SPSS version 24, to find and remove outliers. Thereafter, data were imported to *R* and nested ANOVA's were run for each of the growth parameters using the aov function. Aquaria were nested within replicates which were all nested within treatments with an error term built into the model. For length, four outlier values were removed and for body depth one outlier was removed. For all tests, equality of variance was checked by plotting the residuals against the fitted values; while Shapiro – Wilk normality tests were run to meet the assumption that the residuals approximate that of a normal distribution. Length and body depth data satisfied this assumption ($W = 0.990$, $p = 0.857$ and $W = 0.982$, $p = 0.352$, respectively) while mass data was $\log_{10}$ transformed to conform ($W = 0.975$, $p = 0.128$). Tukey's HSD tests were run to compare differences between treatments and graphs produced on GraphPad Prism 5 were used to display these (Fig. 2).

2.6. Fish survival and plastic ingestion

For each of the plastic treatments and the control, fish were scored with 'zero' for those that survived during growth measurement intervals and 'one' for each mortality incident throughout the experiment. Kaplan – Meier survival curves were then plotted using *R* to compare survival probabilities of the glassfish among treatments during the course of the experiment. Plots were produced using the survminer package for *R*. A log – rank test was also used to determine if overall survivorship differed between treatments. Pairwise comparisons were made using a log – rank test with Benjamini – Hochberg (BH) p-value adjustment. Fish that were culled were excluded from the analysis.

Microplastic particle abundance from fish that died 'naturally', or that were culled, were each correlated with the number of exposure days, to determine if plastics were being accumulated in fish over time. Positive correlations in each case would mean that as the number of experimental days increased, the number of plastic particles found in a fish would increase, giving some indication of an accumulation of particles. For fish that were culled, five individuals from each treatment and five individuals from the control were digested during each measuring interval. Data did not satisfy the assumption of normality and transforming the data did not rectify this. Therefore, Kendall's Tau tests were run on SPSS to determine a rank correlation between time until mortality and the number of particles found within the fish. The treatments were then split and the correlations were run again. A *t*-test was run to determine if the number of ingested particles differed between the plastic treatments for culled fish. Data were $\log_{10} + 1$ transformed to satisfy the assumptions of normality ($W = 0.976$, $p = 0.532$) and homoscedasticity (*Bartlett's* $K^2 = 2.937$, $df = 1$, $p = 0.086$). Control fish were digested in the same way as with the treatments, to observe for any contamination. No particles used in the microplastic treatments were found in any of the control fish that were digested from culled fish and 'naturally' dead fish.

3. Results

3.1. Pilot study of retention

Between the initial exposure and 72 h, plastics in the water column during successive days could either come from being egested by fish that consumed them, or from the remaining 5% of the water left over during the water change. At the start of the retention experiment, all fish were observed to actively ingest plastic particles. The highest concentration of plastic consumed was found during the first 10 min of feeding and was quite variable among the first five fish that were culled (0.002 ± 0.002 g per fish, mean ± S.D.). From the initial dose of 0.05 g, the first five fish consumed a range of 0.06–9.46% of the plastic in each tank with an average of 3.6 ± 3.7%. Thereafter, the mass of plastic found in the fish and the water column decreased considerably, with only a few particles of negligible mass present after 24 h and 48 h, respectively. At the end of 72 h there was no plastic observed either in the water column or any fish (Fig. 1).

3.2. Length, body depth and mass

There was a significant decrease in the standard length of fish in both the virgin and harbour plastic treatments relative to the control ($F = 18.613$, $df = 2$, $p < 0.0005$), while the growth in length of fish in plastic treatments did not differ from each other ($p = 0.679$, Fig. 2a.). Similarly, the body depth of fish in both the virgin and harbour plastic treatments were lower than fish in the control ($F = 24.812$, $df = 2$, $p < 0.0005$, Fig. 2b.). Fish from the virgin plastic and the harbour plastic treatments showed either minimal change or decreased body depth and were not significantly different between each other ($p = 0.147$, Fig. 2b.).

Although the fish in control tanks showed higher mean mass gains than fish in both plastic treatments (Fig. 2c.) and the overall ANOVA was significant ($F = 3.417$, $df = 2$, $p = 0.038$), control fish only differed from the virgin plastic treatment ($p = 0.030$) and not the harbour plastic treatment ($p = 0.391$). Growth in fish mass from the harbour plastic treatment was also not significantly different from the virgin plastic treatment ($p = 0.537$). For all growth measurements, replicates within treatments did not differ among each other (Fig. 2).

3.3. Fish survival and their microplastic load over time

The survival curves for fish from the control and plastic treatments plotted for the course of the experiment, were significantly different overall ($\chi^2 = 7.3$, $df = 2$, $p = 0.027$). At the start of the experiment, all survival curves were similar, however after 50 days the plastic fed treatments showed lower survival probabilities than the control (Fig. 3). However, pairwise comparisons indicated that the survival curve of the control was significantly different from the harbour plastic treatment ($p = 0.026$) but not from the virgin plastic treatment ($p = 0.085$). Fish in the harbour plastic treatments also showed lower survival probability than those in the virgin plastic fed treatments toward the end of the experiment, but there was no significant difference between the curves ($p = 0.490$).

Microplastics were found in 21 of the 67 fish that died during the experiment (31%), i.e. not intentionally culled, from both plastic treatments combined. The average number of particles observed per fish were 0.5 ± 0.86, $n = 30$ and 0.65 ± 1.18, $n = 37$, for virgin and

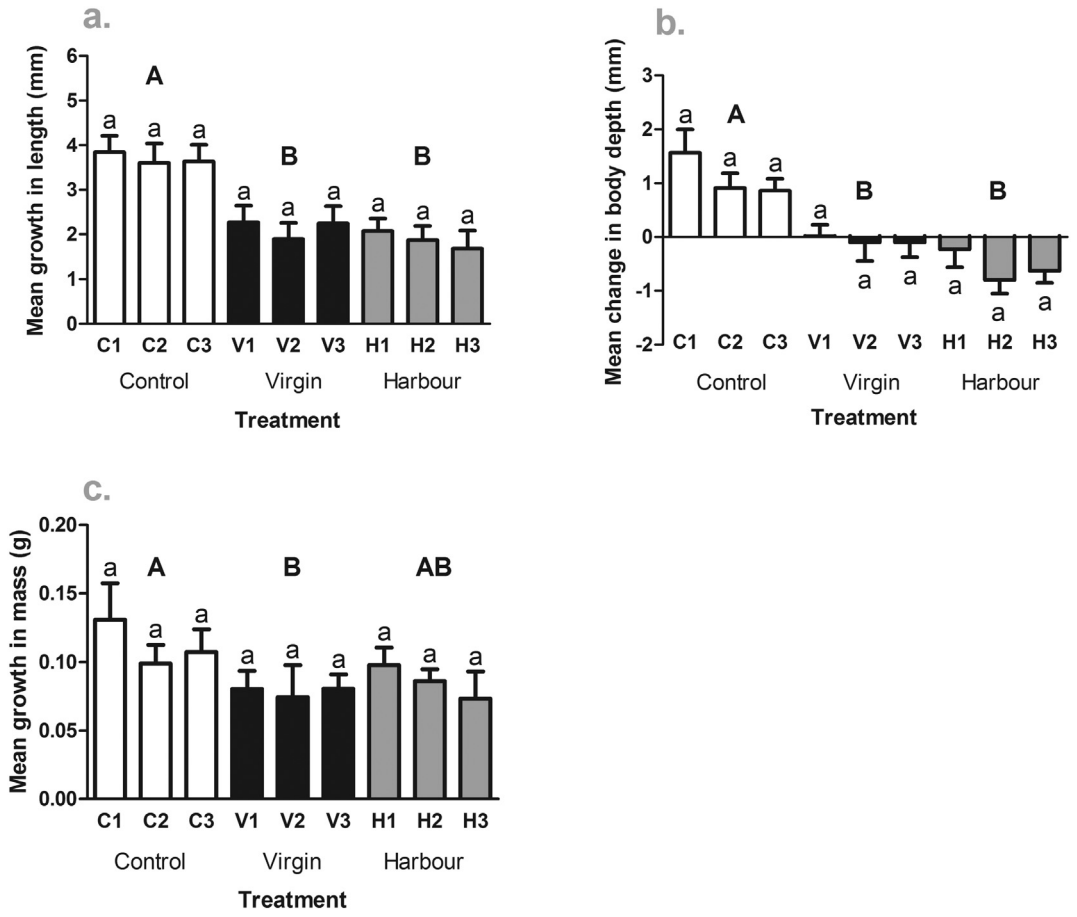

Fig. 2. Mean change in (a.) fish length (mm), (b.) body depth (mm) and (c.) mass (g) for treatments after a three-month exposure to microplastics. Capital letters denote differences between treatments, while letters in smaller case, above the standard error (S.E.) bars, show differences within each treatment. 2 – column fitting image.

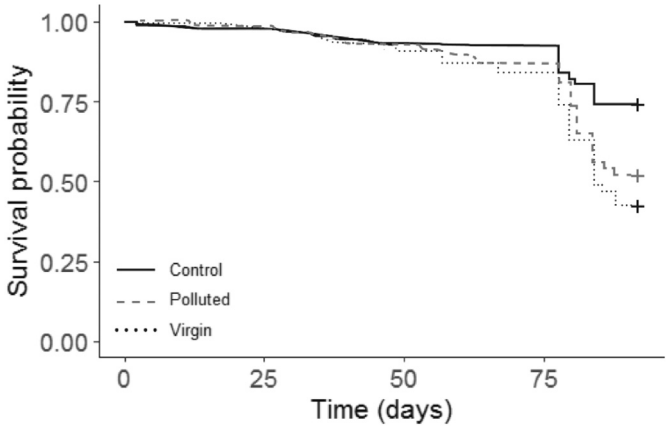

Fig. 3. Kaplan – Meier survival curves for glassfish within virgin plastic and harbour plastic treatments; and a control without plastics. 1.5 – column fitting image.

harbour plastic treatments, respectively. No evidence of a significant correlation between the number of plastic particles and the number of exposure days was found. This held true whether zero values were left out of the correlation (τ *coefficient* $= -0.064$, $n = 21$, $p = 0.719$) or included (τ *coefficient* $= -0.087$, $n = 67$, $p = 0.375$). There was also no correlation found when data were split between harbour plastic (τ *coefficient* $= -0.104$, $n = 37$, $p = 0.436$) and virgin plastic (τ *coefficient* $= -0.093$, $n = 30$, $p = 0.534$) treatments.

Of the 40 culled fish from the plastic treatments, 37 (93%) contained microplastics. The number of microplastics consumed for the culled fish varied considerably. The average number of particles ingested were 29.35 ± 37.59, $n = 20$ and 11.55 ± 11.14, $n = 20$, for fish from virgin and harbour plastic treatments respectively. These were not significantly different ($t = -1.144$, $df = 33.14$, $p = 0.261$). There was a significant albeit weak positive correlation between the number of plastics and the culling date (τ *coefficient* $= 0.333$, $n = 40$, $p = 0.007$). When treatments were split and correlations run, there was a significant correlation between these variables for virgin plastic (τ *coefficient* $= 0.409$, $n = 20$, $p = 0.023$) and no significant correlation for harbour plastic treatments (τ *coefficient* $= 0.237$, $n = 20$, $p = 0.187$).

4. Discussion

4.1. Retention and accumulation

Plastic retention and accumulation is an important consideration when investigating the health effects of microplastic ingestion (Mazurais et al., 2015). The glassfish used in our pilot experiment consumed and egested plastic particles rapidly with limited evidence for long term plastic particle accumulation in fish, even from correlations in the main experiment. This result is common in the literature for fish (Batel et al., 2016; Jovanović, 2017) and oysters (Nobre et al., 2015) and suggests that minimal impact would be caused if isolated particles are incidentally ingested. Particles would have little time to interact with and bring about changes within the fish. This was observed by Mazurais et al. (2015) and Batel et al. (2016) who found no ill

effects on European perch larvae and zebra fish *Danio rerio* (Hamilton, 1822), respectively, during short ingestion experiments, when smooth microspherical particles were egested in a similar amount of time as the glassfish, that egested particles in < 72 h. Retention was also probably influenced by the size and shape of the particles ingested in relation to the organism, since plastic fibres have been shown to intertwine and be retained for months in the lobster *Nephrops norvegicus* (L., 1758), causing physiological changes, such as decreased feeding rates and lowered body mass (Murray and Cowie, 2011; Welden and Cowie, 2016). In addition, increased retention may favour the dissociation and leaching of plastic associated pollutants in the gut, leading to negative health effects (Nobre et al., 2015; Khan et al., 2017).

Although the fish showed low retention, the daily plastic feed ensured a continuous microplastic − fish interaction. This is similar to what may occur in urban harbours, where new microplastics, together with new prey items, are continuously introduced to the system via storm water drains and river outlets (Browne et al., 2010). In this way, longer term studies show organismal changes similar to instances of when particles are retained. For instance, Pedà et al. (2016) found that European sea bass exposed to polluted PVC pellets for three months, had severe changes to their intestinal structure; and after two months Rochman et al. (2013) observed the detrimental effects of microplastic ingestion on liver function. This may help explain why limited changes in growth and survival of glassfish were found during the beginning of this experiment, whilst changes were observed in the longer term.

4.2. Growth

The hypothesis that growth would be adversely affected by the addition of microplastics to the fish's diet was accepted. After 3 months, the treatment fish showed significantly lower growth in length and body depth than fish in controls. They also had a smaller growth in mass, although not significantly so from the harbour plastics treatment. The negative effect that the microplastics had on the growth of the glassfish has also been shown for freshwater fish (Cedervall et al., 2012), invertebrates such as *Daphnia magna* (Besseling et al., 2014) and earthworms (Huerta Lwanga et al., 2016) and was attributed to a compromised energy budget (Lu et al., 2016). The ingestion of microplastic particles has been shown to place an added energy burden on organisms and a decrease in energy reserves through the catalysis of lipids (Cedervall et al., 2012; Wright et al., 2013). In this way, the fish may have had to redirect energy usually used for growth, toward other vital maintenance functions such as ridding the body of plastics and their additives. Coping with other stresses such as inflammation (von Moos et al., 2012) and compromised endocrine system (Rochman et al., 2014), liver function and food absorption (Rochman et al., 2013; Lu et al., 2016), also requires added energy (Wright et al., 2013). With energy used for targeting these sub − lethal effects, decreased feeding (de Sá et al., 2015; Bergami et al., 2016) and a possible false sense of satiation (Cole et al., 2015) can further reduce the energy available for optimal growth. One interesting result was also that fish body depth in plastic treatments remained stagnant and even showed some decrease over the experimental period. A decreasing length is rare but has been shown in juvenile salmoids that have had their nutrition affected under harsh winter conditions (Huusko et al., 2011). There was also evidence of a reduction in feeding during the onset of mortality, since there was a much lower percentage of a plastic in fish that died during the experiment compared to those that were culled. The introduced microplastics were found in fish even toward the end of the experiment, indicating that fish did not avoid it, even after being in the treatment for three months.

It was initially hypothesised that the harbour plastic treatment would be more detrimental to fish, as they may have accumulated organic pollutants (Velzeboer et al., 2014). However, all growth measurements for fish did not significantly differ between the virgin plastic and harbour plastic treatments. Increasing evidence has also shown that organic pollutants may not be as bioavailable to organisms from plastics compared to natural vectors like coal and wood (see Beckingham and Ghosh, 2017). Intrinsic leachates from plastics themselves may therefore be of more concern to organisms than pollutants carried over (Nobre et al., 2015). The mass of fish in the harbour plastic treatments showed no significant difference from fish in the controls, but did differ significantly from fish in the virgin plastic treatment. One possible reason for this may have been that the negative impacts on mass were offset by the additional nutrition provided by biofilms present on harbour plastics, since they were not cleaned before use.

4.3. Fish survival

Survival curves from the harbour plastic treatment were significantly different from the control but not from the virgin plastic treatment, yet both treatment curves fell sharply toward the latter half of the experiment. This showed that with continued plastic supply over an extended period, the probability of fish survival feeding on plastic decreases. In addition to microplastics and their chemical additives being potentially toxic (Nobre et al., 2015), they have also been shown to cause DNA damage (Ribeiro et al., 2017) and can also make fish more susceptible to diseases through a reduced immune system function, which can all impact survival (Greven et al., 2016).

The time of exposure and the concentration of plastic particles seem to govern mortality rates (Mazurais et al., 2015). An example of this is shown in earth worms, *Lumbricus terrestris* (L. 1758), when mortality was higher in 60 day experiments of plastic exposure, compared to shorter two week experiments (Huerta Lwanga et al., 2016). Mortality was delayed when plastic concentrations were lower. However, this is not always the case since Pedà et al. (2016) found intestinal alterations in European sea bass exposed to polluted PVC pellets, in a 90 day treatment yet no mortality was found. Size may have an influence here, since the fish used by Pedà et al. (2016) were much larger than fish used for this study or fish used by Mazurais et al. (2015). This suggests that small juvenile fish could be more susceptible to mortality from microplastic ingestion than larger fish. Since the survival of glassfish in this study was affected by microplastic ingestion, it suggests that microplastic ingestion can have a potential negative effect on their population. However, most fish produce large numbers of offspring in anticipation of high juvenile mortality (Sogard, 1997), and therefore we cannot conclude that population effects will occur.

5. Conclusion

It is concluded that if these glassfish encountered and ingested isolated plastics particles in the field, then it should be rapidly expelled with minimal harm done to the organism. However, when fish are exposed to a continuous supply over longer periods, as in urban harbours, this can have negative effects on growth parameters and survival. This may have consequences for juvenile fish species of commercial importance that use urban estuaries as nursery areas, since models based on decreasing fish body size predict negative effects on fish biomass and yields; and this can also affect food webs by influencing predation (Audzijonyte et al., 2013). Impacts on survival will also directly affect yields and thus can possibly have both economic and ecological consequences. More studies are required to determine if there are population impacts to juvenile fish.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2019.02.037.

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