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Brain Food? Trophic transfer and tissue retention of microplastics by the velvet swimming crab (*Necora puber*).

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Abstract

Knowledge of the fate and persistence of microplastics within the tissues of marine organisms remains poor, despite their ubiquitous nature in marine habitats and ingestion by a range of species being well understood. Mussels (*Mytilus edulis*) were fed 50µl (~ 4.1 x 10^6) of 0.5µm polystyrene fluorescent microplastic spheres. Velvet swimming crabs (*Necora puber*) were subsequently fed either one or three dosed mussels and were sampled after 1 hour, 1, 7 or 21 days post consumption. The stomach, gills, testes and brains were removed and digested. Microplastics were present in all tissues sampled and remained present for the duration of the trial. Only in the stomach and gills was there a decrease in the number of microplastics over time. The stomach and testes showed a significant increase in the number of microplastics present with the number of mussels consumed. The number of microplastics present in the brain remained constant throughout the duration of the trial. Our study is the first to demonstrate the presence of microplastics in the brain of any crustacean species. This has possible implications for a range of behaviours including predator avoidance, foraging and reproduction. Further work is needed to determine whether presence and persistence of microplastics in the brains of crustacean species affects behaviour.

Keywords microplastic, *Necora puber*, *Mytilus edulis*, marine pollution, brain, stomach, testes, gills, bioaccumulation, crustacean

1. Introduction

Microplastics (≤ 5mm diameter Lusher et al., 2013) are now considered a ubiquitous pollutant in marine systems (Eriksen et al., 2014). Their occurrence arises from both primary pollution, via manufactured microplastics (MPs), or as a secondary consequence of degradation of larger plastic pollution (Anderson et al., 2016) and can be in the form of pellets, beads or fibres from a range of materials (Rocha-Santos and Duarte, 2015). As a consequence, ingestion of MPs is thought to be a significant risk to marine organisms (Lusher et al., 2013) and their presence has been recorded in the gut contents of a range of species (de Sá et al., 2018) including True's beaked whale (*Mesoplodon mirus*) (Lusher et al., 2015), the Norway lobster (*Nephrops norvegicus*) (Weldon and Cowie, 2016), Atlantic mackerel (*Scomber scombrus*) (Rummel et al., 2016), and the marine copepod (*Acartia clause*) (Botterell et al., 2019).

The debate around the definition of microplastic and nanoplastic particles is still unresolved with many authors still classifying any plastic particle that is ≤ 5mm diameter as microplastics. Some authors have attempted to quantify the size class of plastics, with different studies classifying an upper size limit of 100nm and 1000nm for nanoplastics

(Gigault et al., 2018). However, for clarity, and based around a lack of certainty pertaining to size classes, any plastic ≤ 5mm discussed here will be termed microplastic.

Ingestion of MPs has been shown to impact both the health and fitness of marine organisms, typically via reduction in feeding stimulus, nutrient dilution and reduced growth (Galgani et al., 2010), but more evidence is appearing to suggest the large surface area:volume ratio of MPs results in high adsorption rates of toxic compounds (Avio et al., 2015), such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), which can bioaccumulate in organisms (McLeod et al., 2015). Significant weight loss has been observed in some species, such as the lugworm (*Arenicola marina*) (Besseling et al., 2013) and langoustine (*Nephrops norvegicus*) (Weldon and Cowie, 2016) due to long gut retention times of MPs which can induce false satiation, reduced feeding times, and stimulation of an inflammatory response in the gut (Wright et al., 2013).

The MPs themselves have also been shown to accumulate in marine organisms within simplified marine food chains. In one study, the trophic level transfer of MPs via consumption of blue mussels (*Mytilus edulis*) and subsequent retention in a number of tissues in the shore crab (*Carcinus maenas*) was observed (Farrell and Nelson, 2013). *M. edulis* is a filter feeder found in the intertidal zone (Tedesco et al., 2010) and is highly efficient at filtering particles from surrounding sea water, including MPs which they retain in the tissue (Van Cauwenberghe et al., 2015). Bioaccumulation of MPs via the food chain could lead to biomagnification. Biomagnification has been shown to occur with toxic compounds, such as methylmercury (Ruus et al., 2015) often leading to concentrations which can impair productivity, reproduction, and survivorship (Galgani et al., 2010; Seixas et al., 2014).

Despite the potential implications of an open circulatory system for crustaceans to enable MP accumulation in tissues other than the gut, there have been few studies examining

MP retention in tissues other than the stomach. No studies to date have looked at MP transfer from the diet of individuals to the cerebral ganglia (brains) in any crustacean species. Recent work on the crucian carp (*Carassius carassius*) has shown 53nm plastic particles consumed in their diet are able to penetrate the blood-to-brain barrier resulting in behavioural disorders, impacting foraging behaviour (Mattson et al., 2017).

Much debate exists over the use of laboratory trials that employ high concentrations of a given contaminant. Ascertaining MP densities in the environment has proven to be highly varied and research in this area is still in its infancy. Environmental levels of MPS appear low. In the North-East Atlantic surface samples revealed MP particle ranges from 0-1.5 particles per m^3 (Maes et al., 2017). Samples taken from the Atlantic Ocean, between Germany and South Africa had MP particle ranges between 0 - 8.5 particles m^3 (Kanhai et al., 2017). MP abundance in the Ligurian Sea in the Mediterranean, was found to be 9.63 particles m^3 (Fossi et al., 2015). It has been noted that studies examining the impacts of emerging contaminants are challenging. This is especially true with regard to MP particle sizes of <100µm (Huvet et al., 2016) and this may account for the relatively low figures quoted in the literature. The use of high concentrations of MPs in ecotoxicological studies should be perceived as a proof-of-concept (Huvet et al., 2016) providing insight into the potential risks of biological interaction. This study was therefore designed to determine whether MPs transferred through a simplified marine food chain can enter, bioaccumulate and remain within, critical tissues of a consumer species – the velvet swimming crab (*Necora puber*).

2. Materials and Methods

Fifty one blue mussels (*Mytilus edulis*) with a mean shell length of 30.62±0.03mm were collected from the Eastern English Channel. Shells were scoured to remove debris and

barnacles that may have retained the MPs. Each mussel was placed into a 400ml beaker with 400ml of aerated seawater. Fifty microliters, equating to approximately 4.1 x 10^6 MPs (Farrell and Nelson, 2013) of polystyrene, Fluoro-max green aqueous fluorescent particles (0.50µm diameter, ThermoFisher Scientific FluoSpheres) were added to each beaker once the shells were observed to open and the siphon exposed. The mussels were left for 90 minutes. During this time the water was observed to change from cloudy, after the addition of the MPs, to clear as the MPs were filtered by the mussels and it is expected that within this timeframe the entire contents of the beaker would have been filtered (Winter, 1973; Schulte, 1975). After 90 minutes the shells were opened with a scalpel and the tissue removed and frozen at -17°C. Three randomly selected mussels were immediately examined under a Leica, DM IL LED fluorescent microscope (Leica, Germany) to confirm the presence of fluorescent MPs in *M. edulis* tissue prior to freezing. No quantification was made of the number of MPs in the tissue of *M. edulis* as these individuals were fed to the crabs.

Twenty-seven male *N. puber* with a mean (± SD) carapace width of 68 mm ± 4.76mm were selected from bycatch retained by a commercial brown crab (*Cancer pagurus*) fishery off the coast of East Sussex for use in the study. This allowed for 3 individuals per treatment at each time point to be sampled, which is the minimum requirement for statistical analyses. This also satisfies ethical consideration of limiting the number of both mussels and crabs used in this type of study. Crabs were maintained individually in isolated seawater flow-through aquariums at 10.8 ºC for the duration of the study. Food was withheld for three days prior to the start of the experiment, after which twelve crabs were randomly allocated into each of two treatments. Crabs in both treatments (n =24) and the control crabs (n =3) were individually fed in buckets of seawater rather than their housing aquaria and were observed to consume the dosed mussels in their entirety. Crabs in treatment 1 were fed the defrosted tissue of a single dosed *M. edulis* and those in treatment 2 were fed three dosed *M. edulis* in

order to determine whether bioaccumulation occurs as MPs transfer. A control group (*n = 3*) were fed untreated *M. edulis*. All crabs were hand fed and consumed all food within two minutes. All crabs were returned to individual seawater flow through tanks and fed a maintenance diet of untreated *M. edulis* on a daily basis for the duration of the trial. Three *N. puber* from each treatment group were sampled at 1 hour, 24 hours, 7 days and 21 days after consumption of the treated mussels. Three control crabs, each fed 1 untreated mussel were sampled at 24 hours. Haemolymph was extracted from each crab immediately prior to to the crabs being euthanised and samples were visually inspected under a fluorescent microscope to confirm the presence of MPs as per Farrell and Nelson (2013). A 5ml syringe (Luer) with a 0.8x40mm needle (BD Microlance 3 Nr.2) was inserted into the 1st arthrodial membrane at the base of each pereopod on the left side of the body. Haemolymph (500µL) was mixed with 1250µl of 20% formalin in an Eppendorf tube.

Two litre beakers were filled with 1L of seawater and ice to aid immobilisation (Gardner, 1997). To this 250µl of clove oil and 250µl of 70% ethanol were added as an overdose of sedative (Garciá-Franco, 1992; Cooke et al., 2004). Individual crabs were left for 1 hour before being removed to record carapace width (mm) and wet weight (g) prior to dissection. Crabs were thoroughly rinsed and the complete stomach, brains, gills and testes were dissected out and placed in individual 15ml Eppendorf tubes with 5ml of 10% formalin. Given the nature of the open circulatory system in crustaceans it is possible that MPs may be present on external surfaces of tissues, therefore, once fixed, the tissue was removed from the formalin and rinsed thoroughly in running water in order to remove both formalin and as many MPs that may have adhered to the surface of the tissues as possible. The tissues were then placed in 15ml Eppendorf tubes with 10ml 2M NaOH. The samples were regularly agitated over an eight week period using sonication to encourage tissue fragmentation and improve the digestive effects of the sodium hydroxide (Stojicic et al., 2010).

Ten subsamples of 20µl (200 µl) of each digested tissue sample from each individual were run through a flow cytometer (Accuri C6 Sampler, BD Biosciences). A 96 well flat bottom plate had 50µl of tissue sample pipetted into each well, with 10 wells per sample. The flow cytometer was calibrated to run 20µl of each sample, recording the level of fluorescence emitted by the MPs. The flow cytometer output was analysed using BD Accuri C6 plus Analysis Software to produce a count of MPs in each sample.

All data was analysed using SPSS Statistics v.24. Analysis of Variance (ANOVA) and Tukey's pairwise comparisons were used to test for significant differences in size of mussels and crabs between treatment groups. Two-way ANOVA and subsequent Tukey's pairwise comparisons, based on the mean flow cytometry counts per individual, with the number of contaminated mussels consumed and time as independent variables were used to identify the determining factors in mean MP occurrence in tissue samples.

3. Results

There was no significant difference in mean shell lengths (overall mean ±SE = 30.62mm ±0.03, $n = 51$) of *M. edulis* dosed with MPs and subsequently consumed by *N. puber* in trials (ANOVA; $F = 1.07$, $df = 15$, $p = 0.471$). The mean carapace width of crabs within each treatment was not significantly different, but there was an observed difference in wet weight between groups (Table 1). However, Tukey's post-hoc analyses did not identify any significant pair-wise comparisons.

No MPs were found in the control group. MP presence in the haemolymph was confirmed via fluorescent microscopy (Figure 1). Two-way ANOVA showed crabs fed three mussels had significantly higher counts of MPs in their stomach tissue, than individuals fed a single mussel ($F_{Mussels} = 6.31$, $df = 1$, $p = 0.023$). Counts for stomach tissue sampled from crabs 1 hour after consumption of mussels were significantly higher than the remaining three

sampling points ($F_{Sampling\ Point}$ = 8.89, *df* = 3, *p* =0.001; Tukeys post-hoc, *p* = 0.003 in all cases, Figure 2A). Additionally, there was a significant interactive effect between the number of mussels consumed and sampling time ($F_{Mussels*Sampling\ Point}$ = 5.27, *df* = 1, *p* = 0.010). MP presence was confirmed in all samples examined and showed no significant difference in recorded abundance between treatments or time points in brain tissue. (Table 2; Figure 2B). Presence of MPs in gill tissue was significantly different over time, but was not influenced by the number of mussels a crab had been fed (Table 2). MP abundance was significantly lower in crab gill tissues sampled at 21 days post feeding than all three earlier time points (Tukey's; 1hr *p* = 0.032, 24 hrs *p* <0.001, 7 days *p* =0.003; Figure 2C). MP abundance in testes samples showed no significant difference between sampling points, but was influenced by the number of dosed mussels the crabs had been fed (Table 2; Figure 2D).

4. Discussion

All tissues sampled from *N. puber* in this study showed not only the presence of MPs immediately (1 hr) following consumption of contaminated mussels, but also the continued retention of MPs to a greater or lesser extent, for the duration of the study. However, due the small sample sizes employed in this study the significant of trends in change of MP occurrence over time and the impact of increasing exposure via consumption of multiple mussels should be treated with caution. Whilst these results largely mirror those of Farrell & Nelson (2013) further work with larger sample sizes is required to fully understand the impacts of increasing dosage on long term retention of MPs. Additionally, this work is the first that the authors are aware of, to identify the presence and retention of these MPs in the brain of any crustacean species.

The previous study on *Carcinus maenas* (Farrell and Nelson, 2013) showed MP uptake into the tissues, likely a result of MPs entering the haemolymph through the

hepatopancreas for transport to the tissues (Wang et al., 2014). However, Watts et al. (2014) observed MPs only present in the hind and foregut gut of *C. maenas* post ingestion and note that they were not translocated to the haemolymph of the crabs, in contrast to the observations in this study (Figure 1). The MP size may have impacted the findings, as the MPs used by Watts et al., (2014) were 20x larger than those used in this study. Translocation of MPs has shown varied results and it would appear that the particle size plays an important role in the fate of the ingested MP particles. Smaller particles are seemingly able to translocate to the tissues more readily than larger particles. In *C. carassius* particle sizes of 53nm were found to enter the brain (Mattson et al., 2017). Zebra fish *(Danio rerio*) fed 5 and 20µm MPs retained the 20µm particles in the gut, but were shown to translocate the 5µm MPs to the liver and gills (Lu et al, 2016). Translocation of both 3 and 9µm MPS were observed in the haemolymph of *M. edulis,* with significantly higher levels of 3µm MP present (Browne et al., 2008). Other studies have shown that the MPS appear to pass through the gut and are egested. Pacific oysters (*Crassostrea gigas*), fed 2 and 6µm MPs did not accumulate plastic particles in the gut, but excreted them rapidly. In addition there was no evidence of translocation of MPS to any other organ or tissue (Sussarellu et al., 2015). No translocation was found in *C. helgolandicus* fed 20µm MP particles, with MPs only being observed in the gut of this species (Cole et al., 2015)

As might be expected, counts of MPs in this study were highest in the gut with the largest numbers observed after one hour of consumption. The crabs that consumed three mussels had over six times the number of beads as those that were fed one mussel. The variation in the numbers of MPs in the gut of crabs that were fed three mussels could have been a result of the feeding mechanism, whereby mussels were torn and shredded prior to consumption, releasing MPs into the water. Residence of MPs in the gut showed a significant decline in presence over time, likely a result of excretion from the individuals, in combination

with transport via the haemolymph to other tissues within the body. In the gills, the significant decrease in MPs counts in the final sampling point might be explained by the ammonia excretion function these structures provide for aquatic crabs (Weihrauch et al., 2004). Within the brain, gills and testes there was no marked increase in the number of MPs present for crabs that consumed three dosed mussels over those that had consumed one mussel. The reasons for this are not clear, but it may be that the excretion rate of the MPs is rapid and that the uptake rate into the haemolymph and tissues is limited due to this. It is possible that given another dose of MPs during the trial that the number of MPs in these tissues would have increased and the potential for this needs further study.

MPs not only cause physiological dysfunction, but the chemical constituents can interfere with normal tissue function (Avio et al., 2015). For example, Pyrene accumulation has been recorded in the Mediterranean mussel (*Mytilus galloprovincialis*) which ultimately led to DNA damage in the form of upregulation of genes that have an important role in cell cycle checkpoints, DNA repair, and cellular responses to agents that cause DNA damage (Avio et al., 2015). Both physical and chemical damage as a result of MP presence indicates the possibility for serious consequences to organ function (Li et al., 2016). Although quantities of MPs introduced to *M. edulis* were higher than are typically encountered in their natural environment (Lusher et al., 2017) there is evidence for tissue permeation and therefore opportunity for damage at low levels of ingestion.

The translocation of MP particles to the tissues and organs of a number of species has been identified. Farrell and Nelson (2013) recorded the presence of MPs in samples of the stomach hepatopancreas, ovary and gills of *C. maenas*, whilst Brennecke et al., (2015) noted the presence of MPs in the gills, stomach and hepatopancreas of the fiddler crab (*Uca rapax*). This study not only detected persistent occurrence of MPs in the stomach, gills and testes of *N. puber*, but determined that the MPs entered and remained within the brain tissue of this

species. The number of MPs recorded in the brain remained constant, showing no significant difference in MP number over the duration of the study. The brain mediates a diverse range of functions, such as olfaction, processing optic information, sensory input, and endocrine regulation. Organisms rely on these functions for behavioural purposes such as locating food resources, finding mates, and predator avoidance, as well as internal regulation (Krieger et al., 2012). The effects of MPs on brain tissue are only beginning to be explored (Mattson et al., 2017), however the results therein suggest interruption of brain function caused by MP bioaccumulation could lead to changes in behavioural and physiological responses.

Behavioural changes could also impair mating by having a direct impact on mating dynamics. This study documents MPs accumulating in the testes of *N. puber.* The discovery of MP presence within the ovaries of *Carcinus maenas* (Farrell and Nelson, 2013) along with the accumulation MPs within the testes of *N. puber*, indicates that both reproductive organs in crabs are able to accumulate MPs. The presence of MPs has been shown to severely disrupt reproductive outputs in the copepod (*Calanus helgolandicus*) (Cole et al., 2015) and reduce the oocyte size and number, and sperm velocity in oysters (*Crassostrea gigas*) (Sussarellu et al., 2015) and it is possible that this could be true in *N. puber*.

The results of this work have important implications for the wider physiological consequences of MP consumption beyond gut retention and suggest the need for further research into the effects of MPs accumulation in brain tissue on the behaviour of impacted crustacean species. Whilst it is acknowledged that the levels of MPs used in this study are not at environmentally relevant levels, the fact that MPs of 0.5µm have been found to translocate from the gut, to the haemolymph and directly to the organs of *N. puber* is cause for concern. This is especially true as *N. puber* are widely consumed and the potential for transfer of MPs to humans is something that needs consideration.

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Ethics: Ethical approval for this research was granted by the University of Brighton's Animal Welfare and Ethical Review Body.

Conflict of Interest. The authors declare no conflicts of interest.

Figure Legends

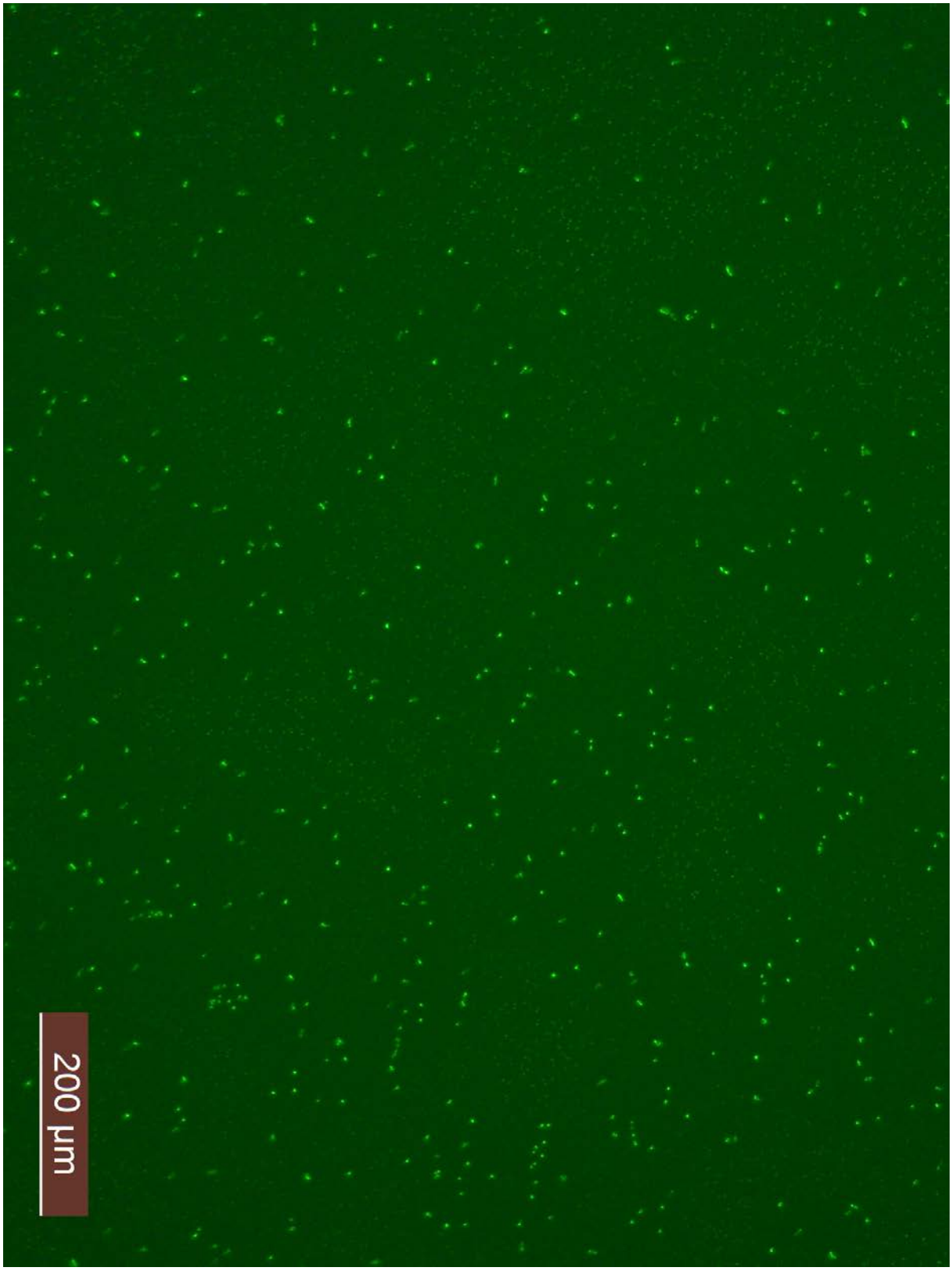

Figure 1. Epi-fluorescent microscope image showing fluorescent microplastic particles in a haemolymph sample collected from a *Necora puber* individual one hour following

consumption of a single *Mytilus edulis* previously dosed with 50µl of 0.5µm polystyrene fluorescent MPs.

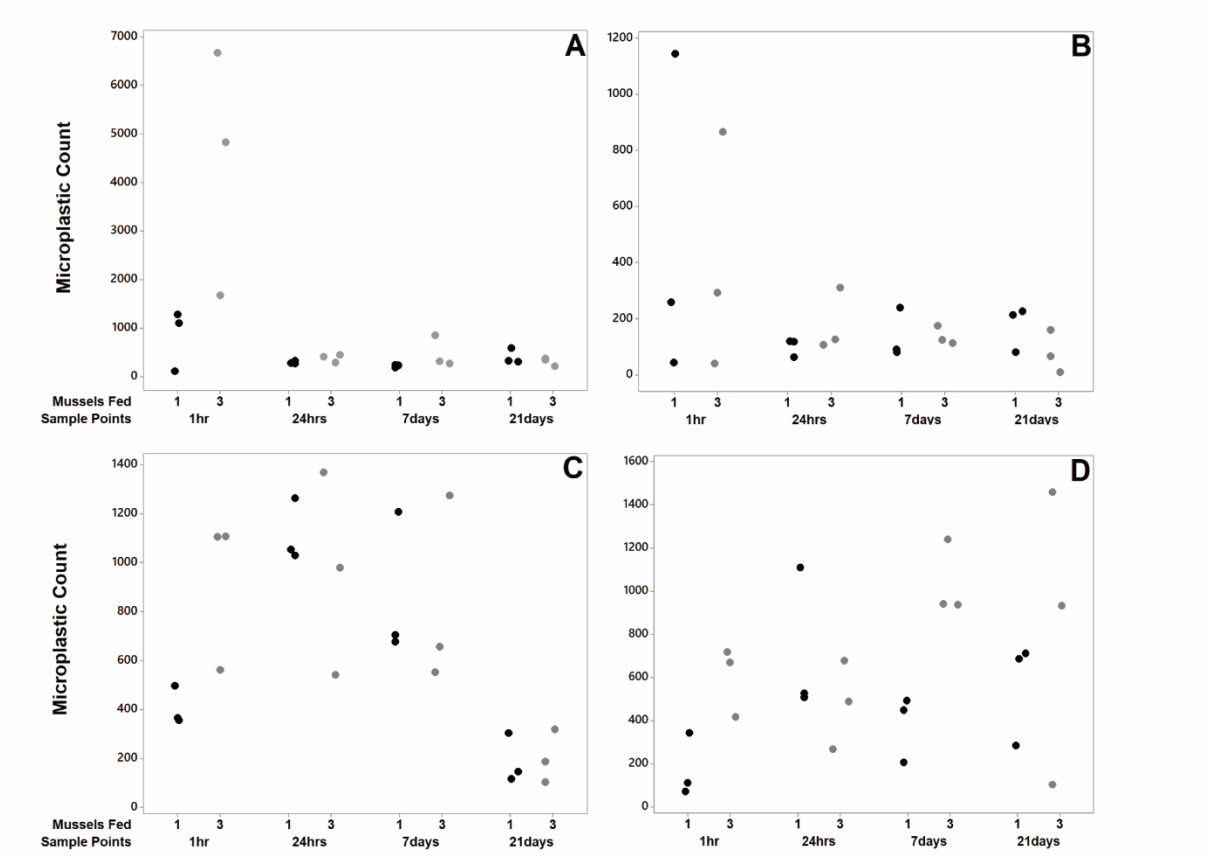

Figure 2. Microplastic abundance in stomach (A), brain (B), gill (C) and testes (D) samples from *Necora puber* sampled at four time points following ingestion of mussels contaminated with fluorescent MPs (n=3 for each treatment at each time point). Black points indicate crabs fed one mussel, grey points indicate crabs fed three mussels (Points have been horizontally jittered to aid visualisation).

Table 1. Mean (±SD) carapace width (mm) and wet weight (g) of *Necorus puber* in each treatment.

	Control	*N. puber* fed 1 *M. edulis*	*N. puber* fed 3 *M. edulis*	ANOVA

		1 hour	24 hours	7 days	21 days	1 hour	24 hours	7 days	21 days	*F*	*p*
Carapace width (mm)	65.38 ±5.40	65.51 ±0.57	64.60 ±0.43	70.46 ±3.51	72.11 ±1.87	68.63 ±0.79	66.81 ±0.54	73.35 ±1.92	72.97 ±0.18	2.16	0.084
Wet weight (g)	73.20 ±13.10	75.95 ±1.92	74.57 ±3.10	98.30 ±13.00	105.60 ±12.80	88.46 ±4.30	73.97 ±1.71	107.40 ±12.90	104.84 ±1.56	2.86	0.031

Table 2. Importance of factors in Two-way ANOVAs examining the determining factors for microplastic occurrence in *Necorus puber* tissues. Significant factors are indicated in each tissue are highlighted in bold.

Source of variation	***df***	***F***	***p***
Brain			
Sampling Time	3	2.03	0.150
No. Mussels fed	1	0.05	0.826
Interaction	3	0.14	0.932
Residuals	16		
Gills			
Sampling Time	3	11.24	**< 0.001**
No. Mussels fed	1	0.64	0.435
Interaction	3	1.89	0.172
Residuals	16		
Testes			
Sampling Time	3	1.35	0.294
No. Mussels fed	1	4.76	**0.044**
Interaction	3	2.19	0.129
Residuals	16		