CURRENT ENVIRONMENTAL MICROPLASTIC LEVELS DO NOT ALTER EMERGENCE BEHAVIOUR IN THE INTERTIDAL GASTROPOD *LITTORINA LITTOREA*

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ABSTRACT

Microplastic ingestion by intertidal fauna is a well-documented phenomenon, with emphasis on the physiological consequences of microplastic exposure. However, the behavioural effects of microplastic ingestion have not been explored to the same degree, even in species with documented microplastic ingestion. In this study, the predator-avoidance emergence response of *Littorina littorea* was assessed and related to microplastic levels within the samples. This is a novel approach to microplastic behavioural experiments, whereby current environmental *L. littorea* microplastic levels are assessed, rather than levels vastly in excess of those recorded under field conditions. The results showed no difference in emergence likelihood or emergence latency related to microplastic abundance. This study shows that microplastics, at their current environmental levels, do seem not affect *L. littorea* emergence behaviour.

Keywords: marine gastropod, predator-avoidance, marine plastic pollution, microplastics, North-Atlantic, Ireland

INTRODUCTION

Microplastics (MPs) are recognised as a marine pollutant of increasing environmental concern, having been recorded in all of Earth's major marine ecosystems (Auta *et al*, 2017). MPs have been shown to be particularly abundant in coastal habitats (Claessens *et al*, 2011; Mathalon & Hill, 2014), which act as marine areas of deposition following transport through rivers and estuaries (Rech *et al*, 2014). Rocky shores may be particularly at risk of accumulating plastic items due to their complex topography, which is also conducive to fragmenting larger plastic items (Hidalgo-Ruz *et al*, 2012, Cole *et al.*, 2011) and entrapping micro debris (Scoffin, 1970). Once MPs enter the rocky shore habitat, they may become available for the species living there, and MP ingestion has now been confirmed for several rocky intertidal taxa including Crustacea (Tosetto *et al*, 2016), Polychaeta (Wright *et al*, 2013), Bivalvia (Green, 2016) and Gastropoda (Gutow *et al*, 2015).

Although there is currently extensive evidence of MP ingestion in intertidal species, the potential implications of this exposure for animal behaviour have not yet been fully assessed and/or understood. One species that has been shown to consume MPs is the intertidal gastropod *Littorina littorea* (Linnaeus, 1758) (Gutow *et al*, 2015; Doyle *et al*, 2019), native to rocky shores of the North-East Atlantic. This species is a voracious grazer of ephemeral green and juvenile fucoid algae. Through this grazing activity, *L. littorea* has been shown to ingest MP particles (Gutow *et al*, 2015), however, little is known about the real-world behavioural implications of this ingestion or whether MP ingestion significantly affects *L. littorea* behaviour at all.

For example, ingestion and retention of non-food items in the gut may increase immune function and lead to a reduction in energy reserves (Watts *et al*, 2015) and thus mobility, while the increased weight burden caused by MP ingestion may also have adverse implications for

mobility (Tosetto *et al*, 2016). Aside from physiological impacts, chemicals adsorbed to the surface of ingested MPs have the potential to bioaccumulate in *L. littorea* (Hartmann *et al*, 2017). These substances may alter chemically mediated behaviour such as conspecific trail following, sensing of dietary cues, and the ability to respond effectively to predation cues, as recently demonstrated by Seuront (2018). The potential negative behavioural implications of microplastic ingestion by *L. littorea* may in turn have knock-on effects for their respective rocky shore community, particularly regarding community structure and dynamics, given that *L. littorea* exert a moderating force on intertidal macroalgae and thus accommodate rocky shore succession (Lubchenco, 1983). Elevated *L. littorea* mortality in response to MP pollution has previously been demonstrated by Green (2016), though it is unclear if this was due to behavioural alterations or the physiological effects of ingestion. Ingestion of MPs by *L. littorea* may also represent a pathway for transfer through the food chain, potentially leading to adverse behavioural implications for organisms at higher trophic levels. Such a mechanism of transfer has been reported between the blue mussel *Mytilus edulis* and the common shore crab *Carcinus maenas* (Farrell & Nelson, 2013). Given that *C. maenas* is the main predator of *L. littorea* on sheltered shores (Hadlock, 1980), it is likely that the same trophic transfer of MPs is also occurring through *L. littorea*.

The aim of this study was to determine whether MP ingestion alters behaviour in *L. littorea*. To achieve this, behavioural trials were carried out to assess the predator avoidance response of *L. littorea* at current environmental MP levels. Emergence is a well-studied response to predator cues in *L. littorea* and occurs when the species emerges from the water to avoid predators. The phenomenon has previously been explored by Hadlock (1980), Jacobsen & Stabell (1999), and Bibby *et al* (2007), with the strongest emergence response usually being elicited by cues from the green shore crab *Carcinus maenas*, the main predator of *L. littorea* on sheltered rocky shores. In this study, emergence likelihood and emergence latency of *L.*

littorea in response to a predator cue (including *C. maenas* cues) was investigated. *L. littorea* emergence likelihood and emergence latency in response to the predator cue was also analysed in relation to sex and shell size.

MATERIALS AND METHODS

Site and sample collection

The study site was a sheltered shore located on the west coast of Ireland, within Galway Bay, on the eastern side of the Mutton Island causeway (53° 15' 42.65" N, 9° 3' 13.703" W; Figure 1). The island hosts a wastewater treatment facility and is adjacent to the River Corrib estuary. The site also runs alongside South Park and is adjacent to Galway City, an urban centre with a population of *c.* 80,000. The selection of this shore took into consideration MPs, which were previously recorded in 58% of the *L. littorea* population inhabiting the study site (Doyle *et al*, 2019). *L. littorea* were collected haphazardly by hand between April and May 2019. Individuals were collected from the *Fucus vesiculosis* zone in the mid-low shore, to ensure consistency with Doyle *et al* (2019). Following transport to the laboratory, samples were allowed a 24-hour acclimation period prior to the behavioural trial experiment. This acclimation period took place in a 20L glass aquarium filled with aerated synthetic seawater prepared specifically for this purpose. Samples were kept for no longer than three days before being deemed unusable as MP retention times in *L. littorea* are currently unknown. Fresh samples were collected each week prior to beginning behavioural trials. In total, 174 individuals were collected. Only *L. littorea* of shell height >12mm were used in the current study, as that is the size at which maturity is generally reached in the species (Williams, 1964; Yamada, 1987).

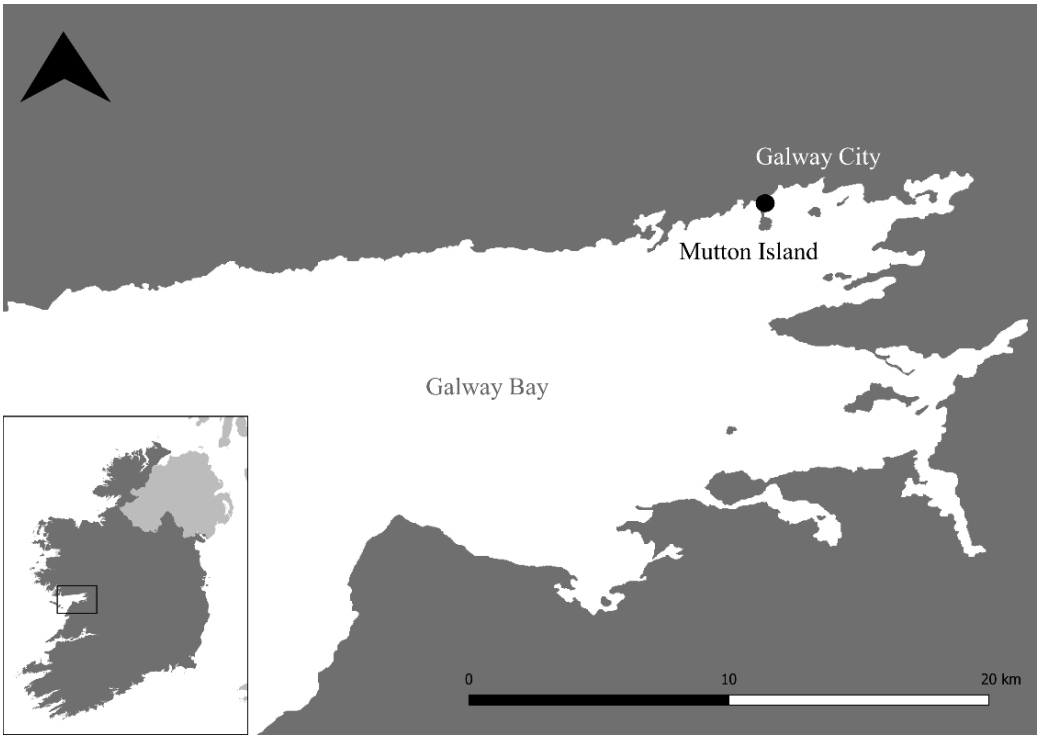

Figure 1: Map of the sampling site location (Mutton Island) within Galway Bay. The location of the bay within Ireland is shown on the inset map (left).

Behavioural trials

To produce the predator cue, one *C. maenas* individual with a carapace width of 58mm was maintained in a glass aquarium with 5L of clean aerated synthetic seawater. Two *L. littorea* had their shells cracked using a pestle and mortar and were added to the aquarium, where they were promptly consumed by the crab. Following a 24-hour period, immediately before beginning the behavioural trials, another *L. littorea* was coarsely crushed and added to the tank with the crab, after which the crab was removed, according to the method used by Cotton, *et al.* (2004). The effluent from this tank served as the predator cue. Prior to each trial, the water in the tank was stirred. Fresh predator cue was produced on every day that trials were carried out with the same crab being used each time, to ensure consistency across trials. Between trials, the crab was maintained in a 60L glass aquarium, filled with clean synthetic seawater. For each trial, six 400ml borosilicate glass beakers were arranged in a row, and evenly lit from above

with a fluorescent light fixture (Figure 2). A volume of 200ml of clean seawater was added to each beaker, to which *L. littorea* were randomly selected and allocated, with one individual per beaker. Individual organisms were placed by hand in the centre of the beakers and a volume of 20ml of treatment was immediately and carefully added to the beakers, making the treatment concentration ~9%. In each series, three individuals received predator cue and three received control (consisting of clean artificial seawater), with the treatment in each series being chosen at random prior to commencing. There was roughly a 40 second delay between the addition of the first and last treatments in each series. Once the final treatment was introduced, the observer left the room to ensure there was no human disturbance to the experiment. Between trials, the beakers were thoroughly rinsed in tap water followed by ultrapure water to remove any trace of prior treatment. Following each trial, the organisms were placed in individually labelled bags and immediately frozen at -20°C to await digestion. In total, the entire experiment took place over the course of three weeks with 29 separate trials, each containing 6 beakers.

Each trial was recorded using a digital video camera (Sony Handycam HDR-CX405), where the video camera was placed on a tripod facing the series of beakers so that emergence at any point in the beaker was visible on the footage. During analysis, two responses were measured. These were emergence likelihood and emergence latency. Emergence likelihood was defined as a binary response consisting of 'emergence' and 'non-emergence', where emergence was deemed to have occurred when the anterior of the snail broke the surface of the water within 900s following the addition of the treatment. Emergence latency was calculated as the time taken for an individual to emerge following the introduction of the treatment. Recording took place in a laboratory specifically fit for this purpose. Recording began prior to the introduction of the treatment and continued for another 15 minutes following the introduction of the final treatment in the series.

Following the experiment, the video footage was analysed by a single observer, to remove possible inter-observer variation. To ensure the experiment was run and analysed blindly, treatment was not visible on the video footage.

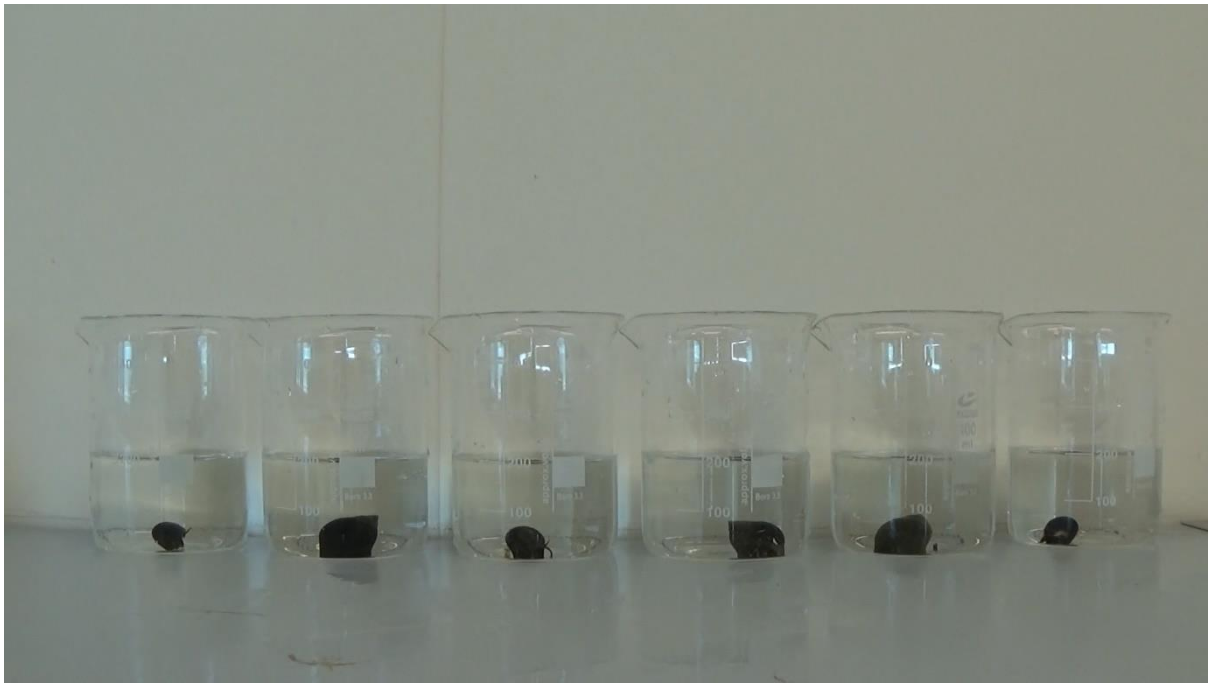

Figure 2 - Video excerpt of a trial showing the experimental design and emergence of *L. littorea* from the beakers. The video is accelerated 10x for brevity and begins following the introduction of the treatment.

Digestion and microscopy

To determine MP levels, individuals were digested according to the method used in Doyle *et al* (2019). In brief, samples were measured and weighed to record shell height and overall weight. Following this, the shell was carefully crushed, and the soft body was extracted, taking care not to break the intestine. Samples were then sexed, and the soft body was weighed, rinsed in ultrapure water, and transferred to small individual glass beakers where they were digested for 24 hours using 10% potassium hydroxide (KOH). All glassware was rinsed in Nitric acid (HNO_3) and triple rinsed in ultrapure water prior to digestion. Following digestion, the samples

were vacuum filtered through glass fibre filters (Whatman grade GF/C). The filters were then transferred to individual petri dishes prior to being assessed for MPs. The filters were assessed for the presence of MPs using an Olympus SZX10 stereo microscope equipped with Image Pro-Plus software (V7). The recovered MPs were classified according to type e.g. nurdle, microbead, fragment, fibre, etc. based on Frias *et al.,* (2018) and Bessa *et al.,* (2019). Airborne contamination was monitored using blank filters, which were left exposed for the duration of the digestions. A random subsample of the recovered MPs (n = 4) were classified using Fourier-transform infrared spectroscopy (FTIR), in order to confirm that the fibres recovered were synthetic in nature. FTIR analysis was carried out using a Brucker Vertex 70V FTIR spectrometer. Spectra for each MP were collected in 128 scans. The recovered spectra were then characterised by comparing them to known spectra from an in-house library.

Statistical analysis

Emergence latency was recorded in seconds (s). Individuals that took longer than 900s (15 minutes) to emerge were deemed to have not emerged and were recorded as such. The first aim was to explore the factors that determined emergence likelihood within the allotted time. To this end, the binary responses of 'emergence' and 'non-emergence' were used as the response variable for a binary logistic generalised linear model (GLiM). Treatment, Sex, and MP abundance were included in the model as predictors, while shell size was included as a covariate. The number of microplastics found in each individual (MPs/individual) was coded as 0, 1, 2, and 3+, to prevent quasi-separation related to small sample size at higher MP levels.

For individuals that emerged, the factors that influenced emergence latency were assessed using a general linear model (GLM). The response variable was emergence latency, Box-Cox transformed in order to achieve the assumptions of the GLM. Treatment and sex were added to the model as fixed factors, while MPs/individual was included as a random factor. Shell

height was included in the model as a covariate. The model was run with a full factorial design. Regression residuals were used to determine if the assumptions of the GLM (normality and homoscedasticity) were met. Analysis was carried out in SPSS version 23 with alpha set at 0.05 for all tests.

RESULTS

A total of 174 *L. littorea* individuals were used in the behavioural trial. Regarding biometrics, shell size ranged from 15.6mm – 29.3mm with an average shell size of 21.06 ± 2.99mm. Fifty-four percent of the *L. littorea* were female while the remaining 46% were male.

The behaviour trial resulted in 135 individuals displaying emergence within 900s, representing an emergence rate of 77.5%. Emergence latency ranged from 101 – 896s. Of the individuals that displayed emergence, 46% were male and 54% were female. Treatment (predator cue or control) was found to have no effect on emergence likelihood or emergence latency. However, emergence as an exploratory behaviour could still be examined in relation to MP abundance, sex, and shell size. The binary logistic GLiM showed no influence of MPs on emergence likelihood (p = 0.799). Treatment and sex also had no significant effect on emergence likelihood (p = 0.236 and p = 0.420 respectively). Shell size was found to have a significant effect on emergence (p = 0.018). To further explore the relationship between size and emergence, a Mann-Whitney U-test was carried out, which showed that *L. littorea* that emerged were significantly smaller than those that did not emerge (p = 0.006).

The GLM results (Table 1) indicated that MPs/individual did not significantly affect emergence latency in *L. littorea* ($F_{3,128}$ = 2.467, p = 0.065; Figure 3). Treatment and sex were also found to have no effect on emergence latency ($F_{1,128}$ = 0.451, p = 0.503 and $F_{1,128}$ =

2.895, $p = 0.091$ respectively). However, size was again found to have a significant effect on emergence latency ($F_{1,128} = 9.979$, $p = 0.002$), with smaller individuals emerging significantly quicker. Normality and homoscedasticity of the standardised residuals were tested using a Kolmogorov-Smirnov test with Lilliefors correction ($p = 0.052$) and a Levene's test ($p = 0.299$). This confirmed that the assumptions of the GLM were met. Additionally, a lack of fit test showed no evidence that the model did not fit the data ($p = 0.204$).

Table 1 - Tests of between-subjects effects showing the analysed predictor variables and their effect on the response variable 'emergence latency (Box-Cox transformed)'.

Tests of Between-Subjects Effects

Dependent Variable: Emergence latency (Box-Cox transformed)						
Source		Type II Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	56.313	1	56.313	234.414	0
	Error	21.368	88.95	0.24		
Treatment	Hypothesis	0.1	1	0.1	0.451	0.503
	Error	28.45	128	0.222		
Sex	Hypothesis	0.643	1	0.643	2.895	0.091
	Error	28.45	128	0.222		
MPs	Hypothesis	1.645	3	0.548	2.467	0.065
	Error	28.45	128	0.222		
Size	Hypothesis	2.218	1	2.218	9.979	0.002
	Error	28.45	128	0.222		

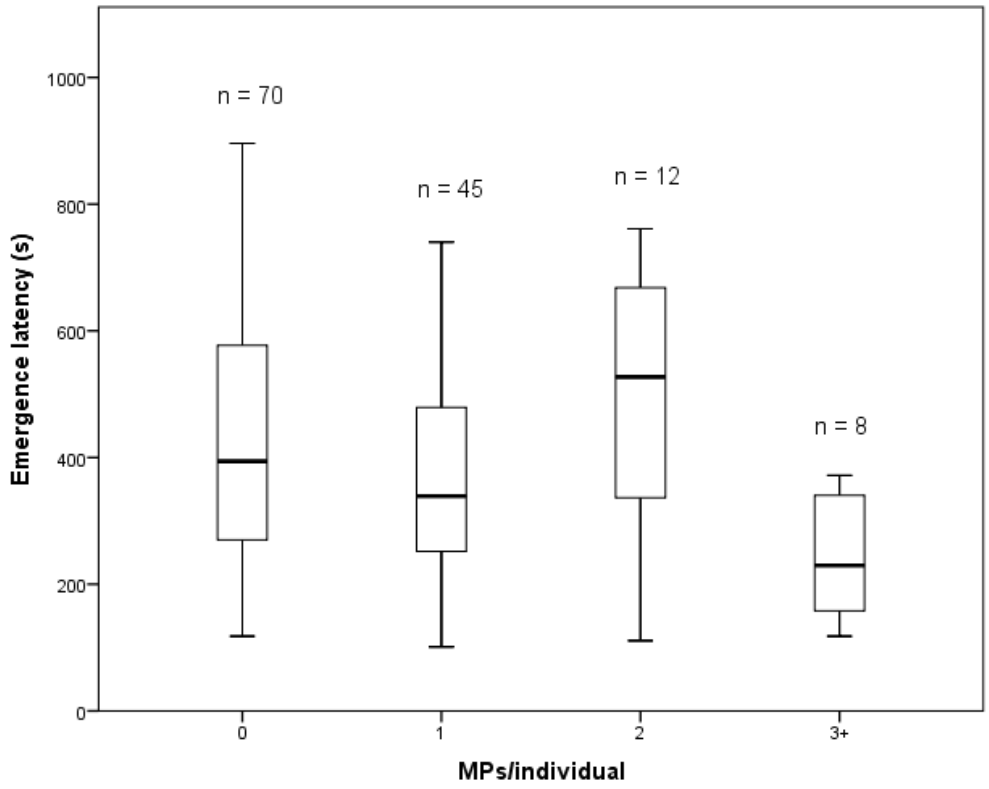

Figure 3 - Boxplot of emergence latency (s) and MP abundance (MPs/individual).

In total, 118 MPs were recovered, ~98% of which were fibres. The remaining 2% of MPs were fragments. The MPs ranged from 106µm - 6420µm in length and had an average length of 1098µm ± 1529µm. The average number of MPs recovered across all samples was 0.68 ± 0.96 MPs/individual, ranging from 0-6 MPs/individual. A non-parametric Mann-Whitney U-test showed this to be significantly lower than the level of MPs that Doyle *et al* (2019) recorded from the same sampling site (2.02 ± 2.07 MPs/individual; $p = <0.001$). The MPs analysed with FTIR were found to be synthetic in nature, with one polytetrafluoroethylene and three polystyrene fibres being identified (Figure 4). The contamination controls showed no atmospheric contamination and so the final results were not altered in any way.

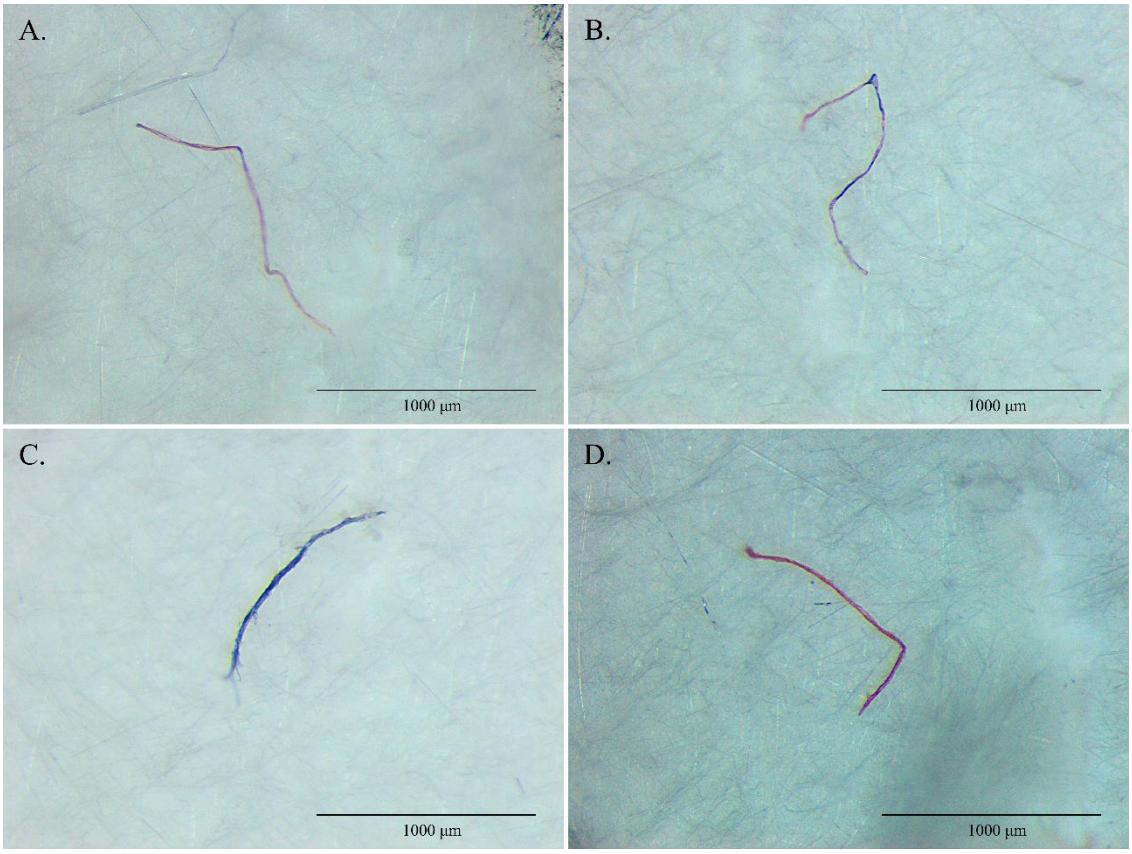

Figure 4 - Microplastic fibres recovered from *L. littorea* following digestion and classified using FTIR analysis (A. – Polytetrafluoroethylene, B., C. and D. – Polystyrene).

DISCUSSION

The *L. littorea* used in the current study showed no response to the predator cue, but rather displayed the same emergence tendencies as the control group. This was unexpected as predator avoidance has been documented in several previous studies using very similar methods (Hadlock, 1980; Jacobsen & Stabell, 1999; Cotton *et al*, 2004; Bibby *et al*, 2007; Seuront, 2018). Though there was no effect related to treatment, behaviour could still be explored in relation to the other predictors, as emergence alone (independent of response to predators) is an important exploratory behaviour in *L. littorea* and is inherently related to mobility.

MPs were not found to affect emergence likelihood or emergence latency in *L. littorea*. This suggests that MPs at their current environmental levels in Galway Bay, which range from 0.59 ± 0.90 to 2.40 ± 2.11 MPs/individual (Doyle *et al*., 2019), are not having a significant effect on emergence behaviour in *L. littorea*. In relation to other taxa, the results presented here are similar to those of Bour *et al.,* (2018), who found that environmentally relevant MP concentrations did not affect behaviour in two burrowing bivalve species (*Ennucula tenuis* and *Abra nitida*). Tosetto *et al* (2017) and Critchell & Hoogenboom (2018) also found no behavioural effect of MP ingestion in the fish *Bathygobius krefftii* and *Acanthochromis polyacanthus*. However, others have observed alterations to behaviour in various species following exposure to MPs. Cole *et al* (2015) found a reduction in feeding behaviour in the marine copepod *Calanus helgolandicus*, whereby the species reduced the amount and size of algae it consumed following MP exposure. Tosetto *et al* (2016) also observed behavioural changes to the beachhopper *Platorchestia smithi* following MP ingestion, namely a reduction in mobility. This reduction in mobility was attributed to an increased weight associated with a higher MP burden. However, this pattern was not observed in the current study at higher MP levels. Green *et al* (2016) also found that *Arenicola marina* produced fewer casts when inhabiting MP laden sediment. These examples suggest that the behavioural effects of MP ingestion are likely to be complex and highly dependent on several factors such as species, body size, MP concentration, polymer type, and chemical load.

Regarding the current study, emergence was the only behaviour explored, and so it is not known if environmental MP levels may be affecting other aspects of *L. littorea* behaviour such as trail following, grazing, or mating. The results presented here contrast with those of Seuront (2018), who found that MP leachate had a significant effect on *L. littorea* emergence likelihood. However, it is possible that the MPs in the present study were not weathered to the same degree

as those used by Seuront (2018) to produce the chemical contaminant. It is also important to note that in that study MPs themselves were not used, but rather their leachate.

Shell size was found to be the only factor that significantly affected emergence likelihood and emergence latency. To the authors' knowledge, this has not previously been shown in *L. littorea*. However, it is not an unexpected result, as the same pattern of size dependent emergence has been demonstrated for other species in the Littorina genus (e.g. *Littorina irrorata*; Stanhope *et al*, 1982). It is thought that larger individuals do not emerge to the same extent as their smaller counterparts, as they are not as susceptible to predation (Stanhope *et al*, 1982).

Comparison of MP levels to Doyle et al (2019)

The MP levels recorded in the current study are significantly lower than those recorded by Doyle *et al.,* (2019). Here, the average MPs/individual is 0.68 ± 0.96, while Doyle *et al* (2019) recorded an average MP level at the same shore of 2.02 ± 2.07. There are several possible explanations for this discrepancy. The data in Doyle *et al* (2019) were collected in November 2017 while the current data were collected in April-May 2019. This may indicate seasonality to MP levels, with potentially more MPs being temporarily deposited from the River Corrib during high flow in the wetter Winter months. A similar pattern was observed by Lima *et al* (2015), in which a salt wedge limited microplastics to the upper estuary of the Goiana river in Brazil during dry months. During wet months, higher freshwater outflow flushed microplastics out of the estuary and seaward. Likewise, Cheung *et al* (2016) observed higher MP abundances during the wet season in the Pearl River estuary in Hong Kong. The reduction in MP levels may also indicate that *L. littorea* are not retaining MPs, but rather excreting them, as suggested by Gutow *et al* (2015). The *L. littorea* samples in Doyle *et al* (2019) were frozen within a few hours of collection. However, the *L. littorea* samples in the current study were kept for up to

three days, during which time some ingested MPs may have been excreted. Most MPs recovered in the current study were fibres, consistent with the results of Doyle *et al* (2019).

Though *L. littorea* emergence behaviour is not affected by current environmental MP levels, if MP levels continue to rise in the future, this potentially may change. Further studies of *L. littorea* at artificially higher MP concentrations may be necessary to determine future behavioural effects.

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