Effects of temperature and microplastic concentration on the consumption of microplastic particles by the detritivore Asellus aquaticus

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Abstract

Microplastic (MP) pollution has now reached a point where particles can be found in every environmental compartment across the globe. However, the ecological and long-term consequences of MPs are largely unknown. These particles are expected to impact many organisms through ingestion, with organisms feeding off particles and detritus predicted to be especially heavily affected. Using the model detritivore *Asellus aquaticus* (Crustacea: Isopoda), the ingestion of MP particles at 3 different temperatures and 3 MP concentrations was investigated, by quantifying the number of MP particles excreted in the animal's faecal matter. I predicted that (i) MP numbers in *A.aquaticus* faecal particles would increase with MP concentration, (ii) MP particle numbers would increase with temperature, (iii) *A. aquaticus* start biomass would not differ between treatments, (iiii) *A. aquaticus* final biomass will not differ from the start biomass, (iiiii) survivorship of *A. aquaticus* will be lower at warmer temperatures and higher MP concentrations, (iiiiii) Increased shedding rates in high temperatures and MP concentrations. The collected data was analysed by dying the samples using Nile Red and counting the MP particles found in the faeces of the animals for each microcosm utilising a fluorescent microscope. An ANOVA analysis was then conducted. This study showed that temperature had no effect on the excrement of MP particles, the only factor of significance was the concentration of MPs in the microcosm. These findings indicate that *A. aquaticus* has little capacity to avoid consuming MPs, rather it is subject to ambient concentrations- the more MPs in the environment- the more it eats. This in turn implies that *A. aquaticus* faecal particles might be a key pathway for the further transfer of MPs in freshwater foodwebs, passed on to further consumers that eat faecal particles. Survivorship, start biomass and final biomass was also analyzed but showed results of no significance.

Keywords: Plastic, microplastic, microplastic pollution, Asellus aquaticus, microplastic concentration, temperature, microplastic ingestion, faecal particles, food web

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Abbreviations

A.aquaticus	Asellus aquaticus
MP	Microplastic
PET	Polyethylene terephthalate
SE	Standard error

1. Introduction

1.1 Pollution and contamination

Since the 1950s millions of tonnes of plastic have been produced, with a large portion of it since finding its way into the natural environment, due to inefficient waste management systems (Barnes et al., 2009). This has led to an increasingly large issue regarding macroplastic, microplastic and nanoplastic pollution in all ecosystems and environmental compartments across the globe (Hale et al., 2020). This includes MPs being found in arctic sea ice, sediments, the atmosphere and the air we breathe (Hale et al., 2020). Hale et al (2020) further stresses the issue by highlighting the ingestion of MPs by organisms in most ecosystems. As MP size reduces due to degradation, the number of individual particles in the environment increases. Thus leading to an increase in the number of organisms able to ingest them, as smaller particles become more freely available to smaller organisms (Peter, 2016). MP consumption affects not only the consuming organisms themselves but also has the potential to affect other organisms and ecological processes further along the food chain. Ingestion can happen in two distinct ways- through primary ingestion and secondary ingestion. Primary ingestion is when plastic particles or larger fragments and items, are consumed directly, normally by mistake. Secondary ingestion is when an organism eats another organism, that has consumed plastics, indirectly eating the plastic through prey (Peter, 2016). Detritivores, such as *A. aquaticus* used in this experiment, often ingest MPs by accident. As the MPs are within the size range of their normal prey (Van Cauwenberghe et al., 2015). The figure below (Figure 1) illustrates how MPs are able to enter the food web through faecal pellets and affect particle feeder species through consumption (Figure: McKie et al., in press).

Particle feeders and other organisms eating detritus are expected to be heavily impacted as their food source becomes more significantly polluted and intertwined with plastic (Peter, 2016). This follows the mechanical degradation of plastic particles into sizes that either are mistaken for food or become embedded in the sediment and detritus on the bottom of rivers, lakes and the ocean (Andrady, 2015). Being exposed to MP particles over a period of time can affect an organism's ability to feed and reproduce, together with changing its metabolic processes and behaviour (Hale et al., 2020). More research is needed within the field in order to understand the biological effects of plastics on the environment (Lechthaler et al., 2020).

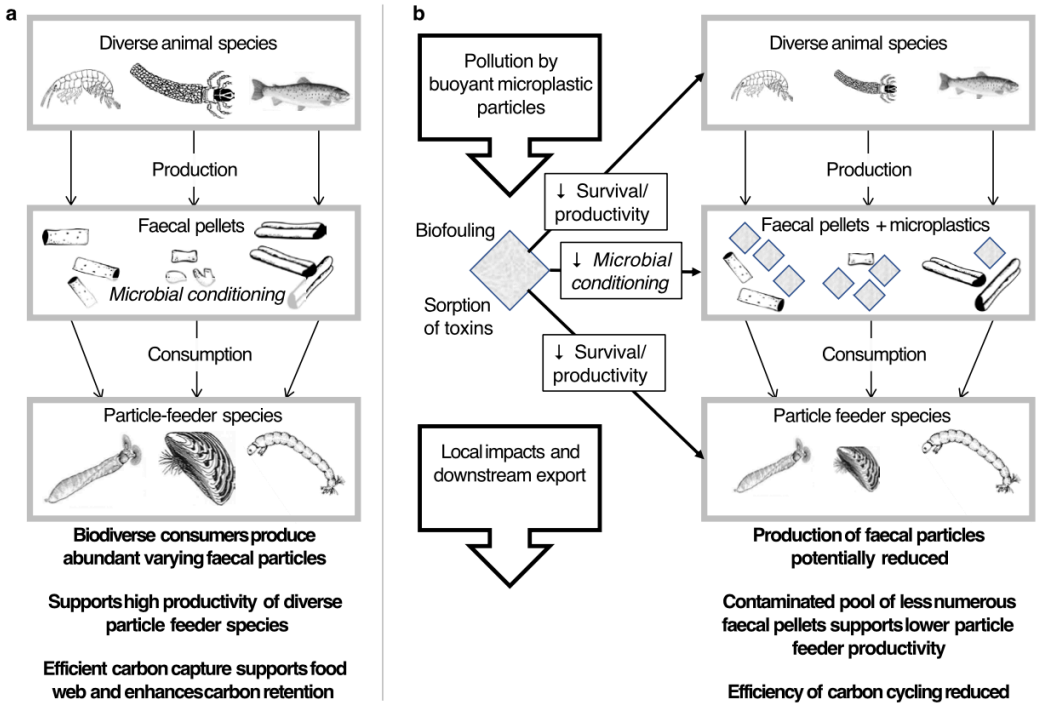

Figure 1

Contamination of a detrital food web with microplastics. Panel (a) illustrates an uncontaminated food web, where diverse animal species produce faecal particles which are colonized by microorganisms and then consumed by diverse particle-feeding organisms. Panel (b) shows MPs contaminating the faecal particle pool, which might have negative effects on microbial conditioning, survival and reproduction of animal consumers (McKie et al., in press).

1.2 Microplastics

Plastics are everywhere in our day-to-day lives, in part due to their low price but also their durability (Thompson et al., 2004). The durability of these long carbon polymer chains used to make plastic is also the main reason behind its persistence in the environment and its ability to withstand biological degradation (Lechthaler et al., 2020). Part of the reason behind this is that plastics contain little nutrients and are largely not water soluble, meaning that microbes that are largely responsible for the degradation of leaf litter, for example, are only able to break down plastics slowly (Zeenat et al., 2021; Novotny et al., 2018). Indeed, according to Zeenat et al (2021), the speed at which microbes are able to break down plastics is determined by how water soluble they are. As a result, the principal way for macroplastics to degrade is by mechanical stress. This mechanical stress could be the grinding of sediments, the movement of waves or UV light, which eventually cause the plastic to fragment into smaller and smaller

pieces (Lechthaler et al., 2020). However, in the absence of UV light, plastics degrade very slowly which often leads to an increased amount being found on the ocean floor or in the sediments of other bodies of water (Andrady, 2015).

MPs are small plastic particles often in the range of 1 to 5000μm. These particles can be pieces of larger fragments called macroplastics that have fragmented through mechanical stress, or beads intended for specific uses, such as in personal care products or in the manufacturing of other plastic products (Hale et al., 2020). MPs that have formed as a result of the degradation of a macroplastic are referred to as secondary MPs while MPs created for a purpose are instead called primary MPs. MPs in many cases are just an interim phase in the degradation process of larger plastic fragments, later becoming nanoplastics smaller than 1μm in size (Lambert & Wagner, 2016). The smaller the plastic fragments become due to degradation, the more readily available they become for ingestion by a large array of organisms (Hale et al., 2020). When more organisms are able to ingest MPs it also opens for the increase of the particles being egested in the faecal matter. Further contributing to the movement and transfer of MPs in the environment via the consumption of faecal particles by coprophages (Kong et al., 2023).

1.3 Asellus Aquaticus

The detrivore *A. aquaticus* is a freshwater isopod which is widespread and found in various locations across Europe (including Scandinavia) and North America (Maltby, 1991). They are benthic meaning they are associated with bottom habitats of freshwater ecosystems and are inclined to live their lives within a small area (Resh, 2008). Benthic species such as *A.aquaticus* are impacted by MPs in sediment as they feed on the bottom of lakes and rivers (Pellini et al., 2018). Their diet is predominantly made up of decaying vegetation, algae, particulate detritus, and other small invertebrates. (Graca et al., 1993). *A. aquaticus* are sensitive to exposure to high temperatures which in time also affects their growth, rate of survival and ability to breed (Di Lascio et al., 2011). They are, however, tolerant of poor water quality and organic pollution, making them a common subject for experiments (Maltby, 1995). There is a high variability of size in adult individuals throughout the habitat range, though males tend to be larger than females of the species (Adams et al., 1985). Some studies have shown, however, that the size of adult individuals is related to the habitat where they are found. It has also been shown that larger individuals are more commonly found in habitats with clean water compared to habitats with polluted water (Maltby, 1991). Subsequently, smaller individuals are more commonly found in areas with periods of higher temperatures (Aston and Milner, 1980). The size of the individuals may affect their ability to withstand pollutants and stressors within their habitat, something

that should be taken into consideration when selecting individuals for scientific purposes (Kiffney and Clements, 1996). *A. aquaticus* sheds its skin to enable growth but has also been shown previously to increase its rate of moulting in response to exposure to pesticide contamination (Dawoud, 2011), as observed also for other freshwater invertebrates (Song et al., 1997).

1.4 Impact of climate change on microplastic consumption

MP pollution is only one of a large number of global changes impacting ecosystems around the globe (Villarrubia- Gómez et al., 2018). The largest of these global-scale problems are climate change and global warming. Research has, however, recently shown how different environmental problems can interact, leading to a stronger impact on biodiversity and ecosystem functioning than if only one was present (Orr et al., 2020). This includes the possibility of MP pollution and climate change interacting (Kratina et al., 2019). The warmer temperatures affect the metabolic rate and therefore the energy balance and behaviour of organisms (Volkoff & Ronnestad, 2020) which in many cases can lead to an increased feeding rate to ensure survival. This is especially relevant for consumers. It is difficult to predict how much of an effect increasing temperatures will have on the said consumers as it is entirely dependent on their thermal preferences. This is according to the thermal equilibrium hypothesis by Ward & Stanford (1982), which states that the metabolic rates, growth and reproductive success of organisms are optimised at the temperature range to which they are adapted. According to this hypothesis, the metabolic rate of organisms declines at both higher and lower temperatures outside of their optimal range. Given feeding rates are correlated to an organism's metabolic rate, this could imply that the uptake of food- and therefore MPs could be highest within the organism's preferred temperature range. Since the rate of feeding is linked to the organism's metabolic rate it can also be assumed that the intake of food- and accidental uptake of MPs, would be highest within the optimal temperature range for the animal.

These factors could, in turn, contribute to an increased rate of MP particles finding their way into the food web, as the feeding rate increases. Especially for particle and detritus feeders who either live in an environment where MPs tend to collect or have a food source that is very similar in size (Peter, 2016). This is, however, largely dependent on if the animals in the food web are able to differentiate between patches in the ecosystem containing MPs or not. If the animals are subject to the MP particles and unable to differentiate between patches containing them, the organism is expected to ingest more MPs (Mateos-Cárdenas et al., 2022).

This might lead to more faecal particles containing MPs. Furthering their transport and movement in the environment via the consumption of faecal particles by particle-feeding organisms (Kong et al., 2023).

1.5 Objective of report

The objective of the report is to quantify the excretion of MP particles in the faecal matter of *A. aquaticus* in three different MP concentrations and temperatures.

1.6 Questions

- Does temperature affect the uptake of MP particles by *A. aquaticus*?
- Does the mean biomass of *A. aquaticus* differ before and after the treatments?
- Does the start biomass of *A. aquaticus* affect the amount of MPs found in faecal particles for each treatment?
- Is there a relationship between the amount of MP particles present in the dishes and the amount of particles found in the faeces of *A.aquaticus*?

1.7 Hypothesis

A. aquaticus start biomass
The mean of *A. aquaticus* start biomass is expected not to differ between the temperatures and MP concentrations. If this is the case, it shows that the distribution of animals in the dishes was not random.

A.aquaticus final biomass
I expect no change in the biomass of *A. aquaticus* at the end of the study, since the time period was too short for significant growth, unless there was mortality in the microcosms.

A.aquaticus mean survivorship
Survivorship of *A. aquaticus* is expected to be reduced in microcosms with warmer temperatures (greater thermal stress) and higher MP concentrations (due to physiological stress associated with contamination of their food supply).

Total MP count
The expectation is that the number of MPs found in the faecal matter of *A. aquaticus* increases with the increasing concentration of MPs added. It is also

expected that temperature will increase the occurrence of MPs in faecal particles, due to increased feeding and excretion rates at higher temperatures.

A. aquaticus exuviae
Increased stress is expected to increase skin-shedding rates, both in terms of increased temperature and increased MP concentration.

2. Material and method

2.1 Method overview

This study looked at the consumption of MP particles by *A.aquaticus* in a factorial microcosm experiment within three temperature-controlled cabinets. The three cabinets were set to one of three temperatures, 16C°, 19C° and 22C°. The microcosms used consisted of 45 glass crystallisation dishes. They contained sand, food and 3 *A. aquaticus.* The dishes were marked with the treatment applied, both for temperature and for MP concentration together with a block letter. M1 dishes were to contain no plastic and become the control, M2 dishes to contain low concentration and M3 to contain a high concentration of MPs. T1 represented low temperature (16C°), T2 medium temperature (19C°) and T3 high temperature (22C°). In each temperature cabinet, 15 dishes were placed, representing 5 from each MP treatment. The blocks used were A, B, C, D and E. These were used in order to avoid mixing dishes up and to allow easy identification. Each block also represented a shelf in each of the cabinets.

2.2 Experiment procedure

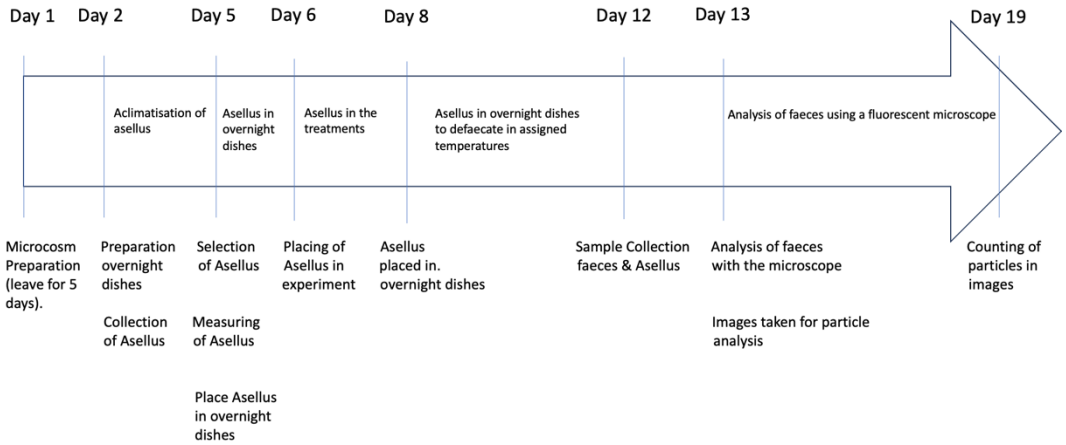

Figure 2

Timeline of the experiment, showing the main steps involved in setting up the experiment, exposure of *A. aquaticus* to the treatments and faecal particle collection and post-feeding trial steps.

The subjects used in this experiment were individuals of *A. aquaticus* collected from a small pond behind SLU campus Ultuna, Uppsala (59.808993N, 17.667064E). The pond was fringed by a small amount of trees on one side and a field and small platform on the other. This was done by collecting leaf matter using a fine-meshed net (1mm). The net was then emptied onto a small platform by the pond and the leaf matter was looked through. The *A.aquaticus* found amongst the detritus were picked with tweezers. They were stored in a plastic container filled with pond water in order to adjust to the inside laboratory conditions and later moved to a glass holding aquarium containing leaf matter and pond water. The 45 glass crystallization dishes (300ml) were then rinsed and prepared for the experiment. They were marked with the concentration of plastic together with the temperature and a letter to mark the block in which they were to be stored in the temperature cabinets.
Then 27g of sterile, autoclaved sand (Fontainebleau Sand, VWR) was added to each microcosm, together with 0.067g of food. The sand which was used had a grain size of 66-177 micrometres. The food was made up of a mix of Tetra Phyll fish food (Tetra, Germany) and ground alder and birch leaf litter. This was done at a 1:1:2 ratio in accordance with Kong et.al (2023).

MP: preparation and addition to microcosms

MP solutions were made by mixing 0.450mg and 6.095mg respectively of polyethylene terephthalate (PET) MP fragments, which were between 25-63 micrometres in size, with 50ml of 90% ethanol in large test tubes. The fragments were prepared prior to this study by grinding larger pellets in a ball mill. These signified the low and high concentrations used in the experiment. The concentrations used represent the sediment concentrations found in locations around the world. The low concentration is the median concentration found in nature around the world, while the high concentration is the highest found in nature (Kong et.al., 2023) The solutions were suspended using an ultrasonic water bath. Then 0.177 ml of the low-concentration MP solution was pipetted into all M2 dishes, this was done with the assumption that the amount of MPs in the solution was 0.009mg/ml of solution. The next step was to pipette 1.307ml of the high-concentration solution into all M3 microcosms, assuming the number of MPs was 0.1219mg/ml of solution. A control containing 0.8 ml of 90% ethanol was added to all M1 microcosms. The microcosms were then left for a period of a few hours in order to allow the ethanol to evaporate. The next step was to add 200ml of pond water (collected prior to the experiment) to each dish. The microcosms were later placed into their assigned temperature cabinets and left for 5 days in

order to culture. Notes were taken regarding the placement of each dish in the cabinets and the water level was marked for each microcosm.

A. aquaticus: pre-experiment gut clearance and size measurement

Another set of 45 dishes were prepared to allow *A.aquaticus* to empty their guts prior to the experiment (hereafter "gut clearance dishes"), these are later used again at the end of the study. These dishes contained nothing but 250 ml of de-ionized water and pond water, both 50% of the total amount of liquid. *A. aquaticus* were removed from their holding aquarium after a few days by gently removing each leaf from the aquarium and removing *A. aquaticus* with tweezers. The discarded leaves were placed in a container with tank water and the *A. aquaticus* in a separate glass container. The water in this container was 50% holding-tank water and 50% de-ionized water. The *A. aquaticus* were then placed in the gut-clearance dishes that were previously marked. They were placed 1 at a time into the dishes until the total amount of *A. aquaticus* per dish was 3. Individuals that were lighter in colour were avoided and those mating counted as 2 individuals. Efforts were made during the selection of individuals to ensure even size distribution among the replicates. Each *A. aquaticus* was then measured with the naked eye using squared paper, where each square was 5mm in length. The paper was placed underneath the glass dishes and the size was quantified and noted for each individual.

Addition of *A.aquaticus* to experimental microcosms and faecal pellet collection

Around 24 hours later the *A. aquaticus* were placed in their corresponding microcosms within the temperature cabinet. This was done with the use of a metal spoon that was wiped with a paper cloth between each animal transfer, with care taken to avoid dipping it into the cultured dishes, and to remove as much water as possible from the spoon while moving the individuals between dishes. The water level was checked for each of the dishes and, if necessary, filled up to be in accordance with the water level prior to the introduction of the *A. aquaticus*. For this purpose de-ionized water was used. To avoid patches without sand forming in each dish this was done slowly and carefully. If patches did form, the dish was carefully swilled around until the patch was once again covered. The experimental microcosms were maintained for 48 hours, giving time for *A. aquaticus* to feed.

The gut-clearance dishes used previously were rinsed with de-ionized water. After 48 hours, the microcosms were removed a few at a time from the temperature cabinets, and the *A. aquaticus* was transferred to the gut-clearance dishes again. This time the gut-clearance dishes were filled with 250ml of de-ionized water.

The *A. aquaticus* were gently scooped out of the microcosms using a spoon and placed on a paper towel. They were then rinsed with de-ionized water and placed into the gut-clearance dishes. Notes were taken regarding observations of the *A. aquaticus* in each dish, such as if they had eggs, had shed skins (exuviae) or if the dish contained any dead individuals.
The gut-clearance dishes were then placed in the exact places occupied previously by the corresponding experimental microcosms in each of the temperature cabinets. They were left for four days to enable the *A. aquaticus* to empty their guts. After the allocated time period had passed the dishes were collected and taken to the lab to begin analysis. Once again notes were taken regarding observations of each dish, such as mortality, exuviae and amount of individuals with eggs. The *A. aquaticus* in each dish were collected using tweezers and placed into a marked test tube, showing the concentration of MPs, temperature and block that the dish came from. All *A. aquaticus* from the same dish were placed in the same test tube. The test tubes were then filled with enough 70% ethanol to cover the *A. aquaticus*, in order to kill and preserve them.

The faecal particles together with skin and other debris were removed from the gut-clearance dishes and placed in a different marked test tube with the dish name. This was done using a plastic pipette. As much water as possible was removed from each test tube using the same pipette. A new pipette was used for each dish to avoid cross-contamination of the samples.

Quantification of MP in the faecal particles

Nile red (Apollo scientific; Code: Bin0465) was mixed together with acetone to give a concentration of 1 µg/mL, which was used as the dye for the plastic particles in the samples (as in: Shruti et al., 2022). The dye solution was made and stored in the dark until being used. 200µl of the solution was then added to the test tubes that were being analyzed that same day using a pipette. These samples were then vortexed and left for 20 minutes, in order to allow the dye to colour the plastic particles. After the 20 mins had passed a slide was made for one sample at a time, using 20µl of the sample together with 10µl of de-ionized water. The whole slide was observed using a fluorescent microscope and images were taken of particles of areas suspected to contain MP particles. A series of 8-12 images were taken per slide at 10X magnification (Images 2 and 6). This image series contained images using BF (bright field), DAPI, FITC, and TRITC in order to allow the identification of the particles (Images 1-8 show 2 different image series). DAPI produces images that have a blue light and picks up the dye that was used on the MPs, (Images 2 and 6) FITC shows organic matter in green (Images 3 and 6) and TRITC that picks up everything, including visible light (Images 4 and 8). The images taken were then analyzed and the plastic particles

were counted using a fixed set of criteria. The said criteria were decided after inspecting reference images of MPs (such as the MP-only ones below) and food particles. This led to the criteria of the MPs having a strong fluorescence in DAPI (blue light), being within a certain size range (25-63μm) and having a physical appearance that is characteristic of MPs. The particles that had unclear appearances were then discussed with the supervisor in order to aid in identification. Each sample and image were noted together with the number of particles found. The total amount of particles for each image was also concluded and noted.

Sample T3M3 D

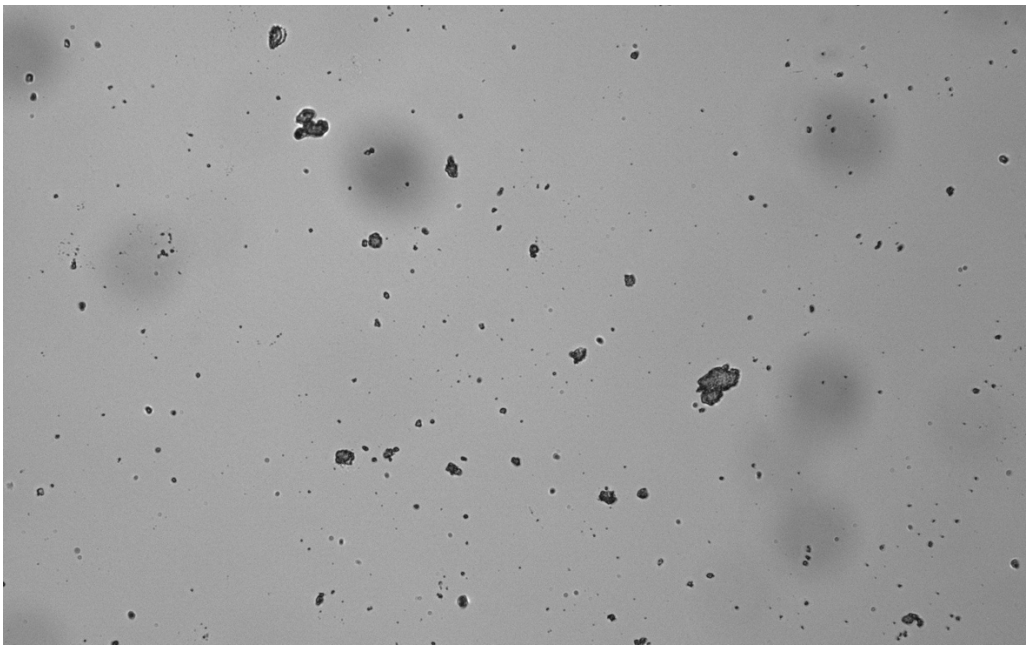

Image 1
Illustrates particles fulfilling the criteria in regards to shape, size and overall appearance. The image taken in brightfield (BF) of sample T3M3 D.

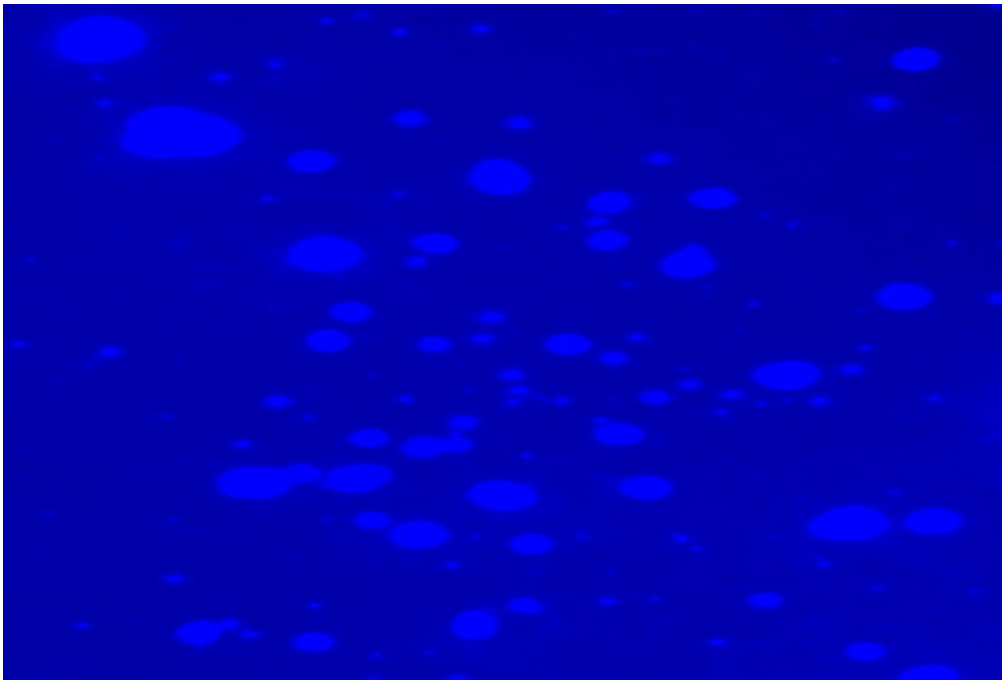

Image 2

The image was taken using DAPI in the same location of sample T3M3 D as the bright field image above. DAPI picks up the dye used for the MPs. The picture shows how the MP particles light up in this specific wavelength of light, aiding identification.

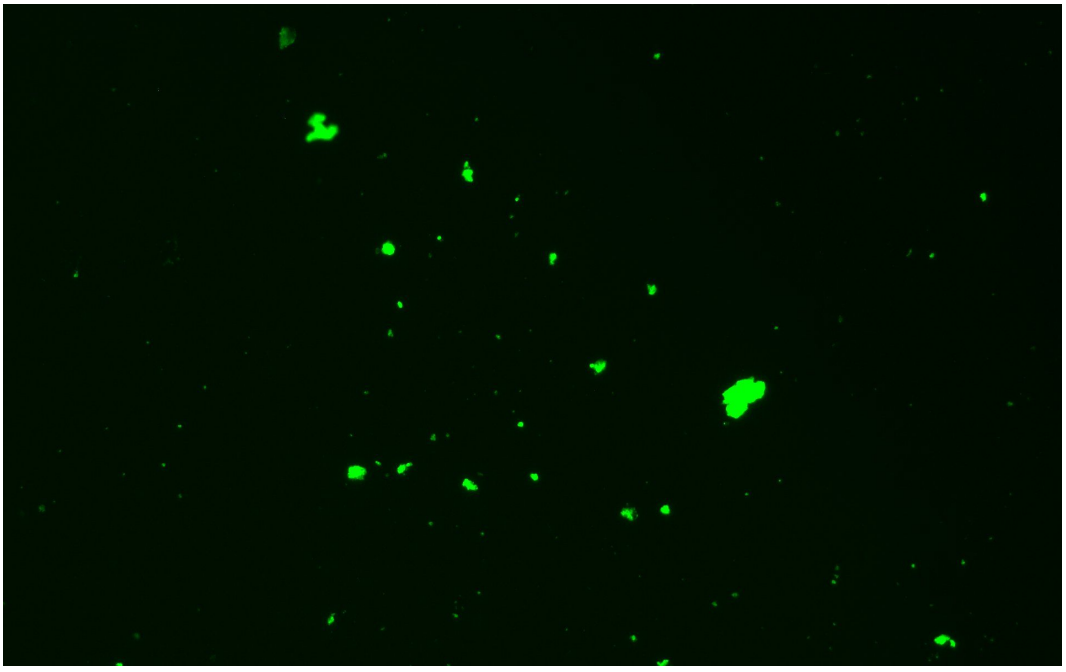

Image 3

The picture above was taken using FITC, which picks up some organic matter. The image if from the same place on the slida as the others from T3M3 D.

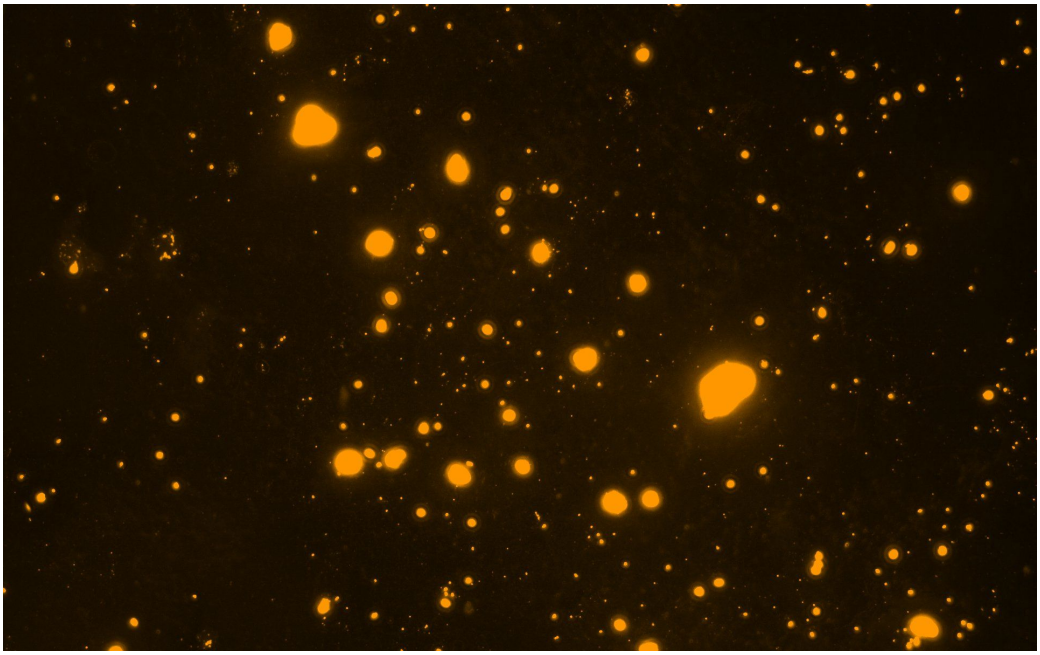

Image 4

TRITC was also used as an identification tool for the MPs, as it lights up everything, including visible light. The image is from the same location on sample T3M3 D as the BF and DAPI images above.

After consulting all images taken in this specific location of sample T3M3 D, 10 particles were identified. The criteria mentioned above were applied and some particles were not counted as they didn't fulfil the criteria of size and shape.

MP only comparison sample

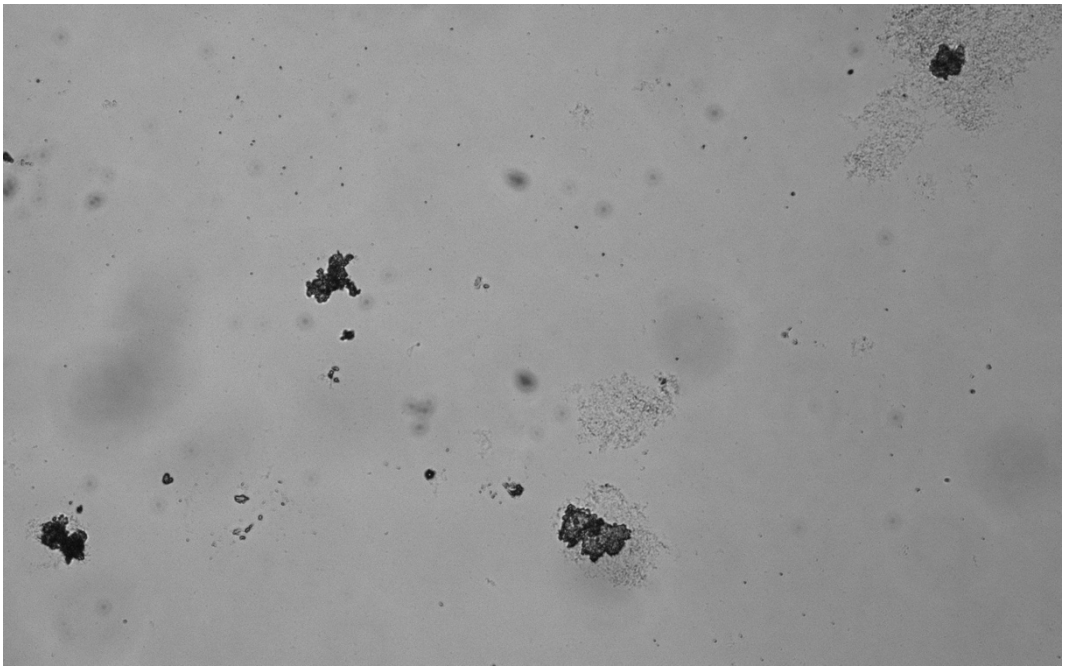

Image 5
MP only comparison image taken in brightfield (BF), showing particles assumed to be MPs.

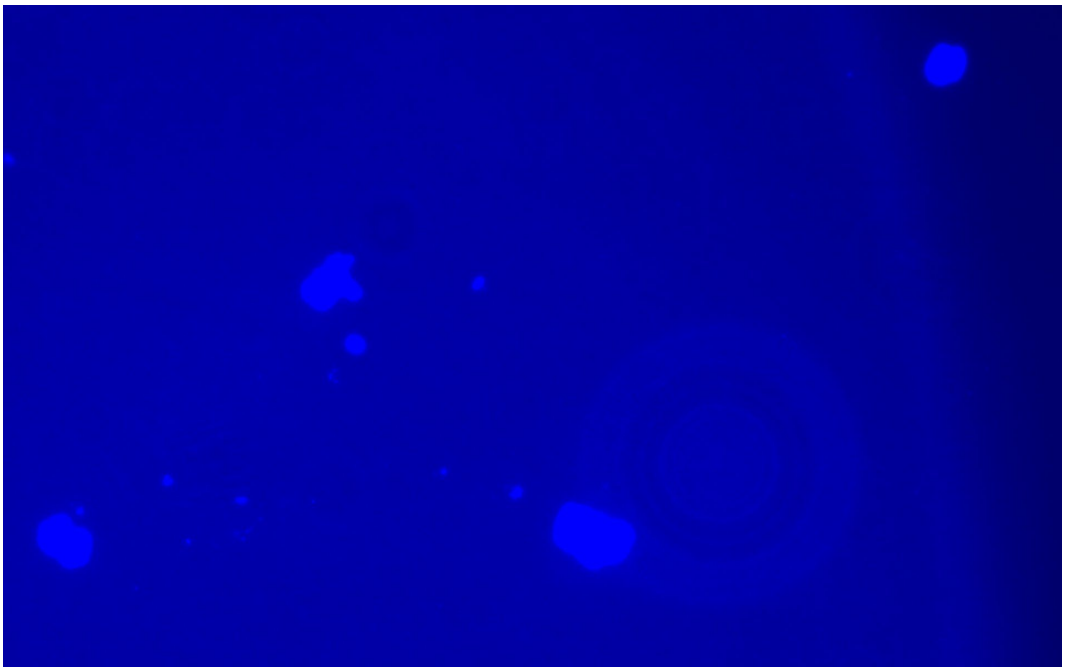

Image 6
MP only comparison image taken in DAPI, in the same location of the sample as the BF image above.

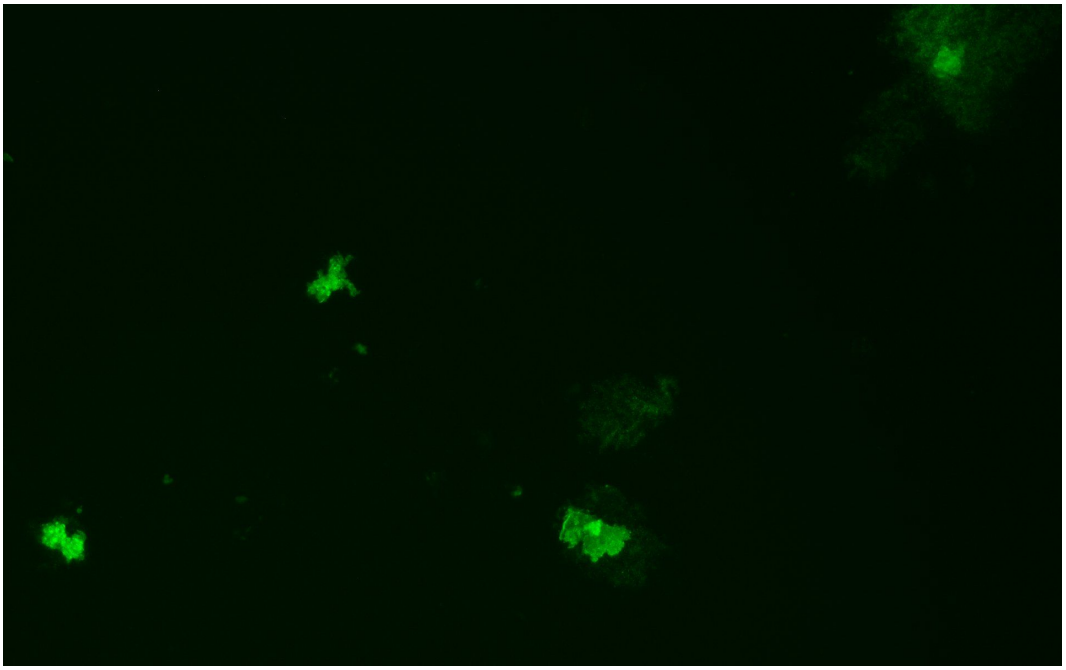

Image 7
Image taken for MP comparison purposes in FITC. Captured in the same location as the others shown for MP only.

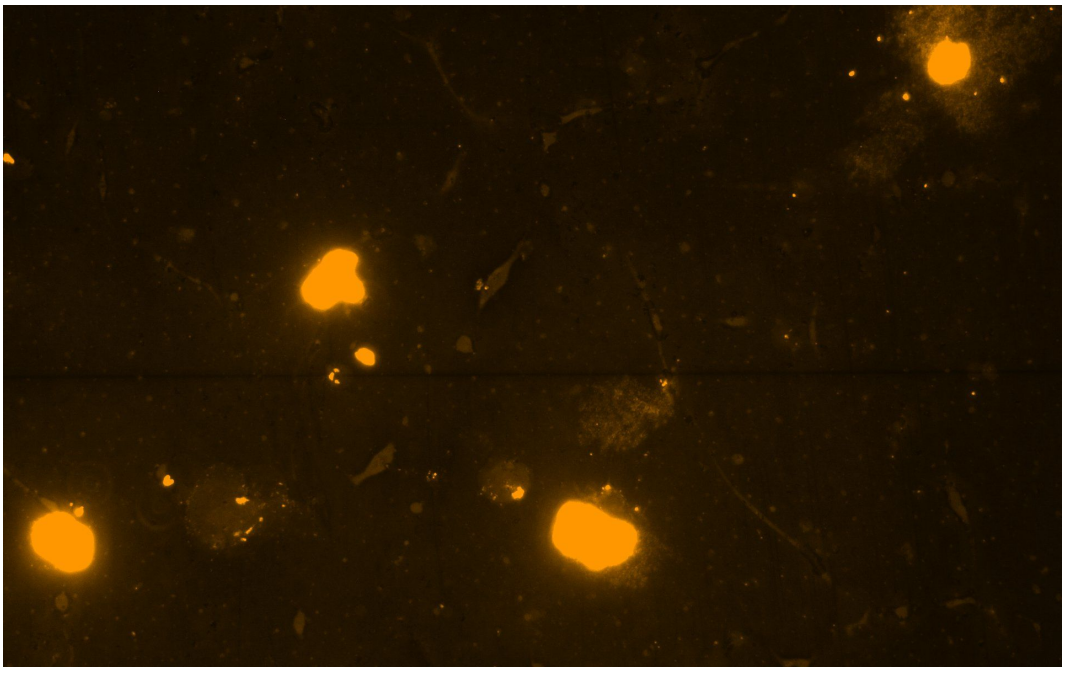

Image 8
Image was taken of an MP only sample in TRITC, in the sample spot as those above.

These MP only images were used in order to evaluate if the particles lighting up in different wavelengths were MPs, in the samples. In the images shown, 4 MP particles were identified.

2.3 Data analysis

The data analysis was conducted using JMP Pro 16 (SAS Institute). I used random block analysis of variance to assess the effects of temperature and MP concentration on *A. aquaticus* body size, survivorship, and on MP particle counts. The ANOVA analysis (Analysis of Variance), which was conducted for temperature and MP concentration as fixed factors and blocks to account for random effects, was done in order to account for background variation in the blocks. The data collected for exuviae was only shown graphically as the distribution was not suitable for an ANOVA analysis. Assumptions of Data were either log or square root transformed in order to meet the assumptions of ANOVA

(constant variance). These assumptions were checked using plots of residuals against fitted values, shown in Appendices 1-4.

The mean survivorship of *A. aquaticus* was used in calculating the start and final biomass for each treatment. Specifically, *A.aquaticus* biomass was calculated prior to the data analysis in JMP pro by using the formula by Franken et.al (2005), where the length of the individuals was multiplied by the number of individuals in a treatment. The biomass was also calculated using the same formula after the treatments to account for changes in the total biomass caused by the death of individuals.

Since biomass might influence particle consumption and excretion, I conducted additional ANOVAS for particle count data standardized for initial and final *A. aquaticus* biomass.

3. Results and discussion

3.1 Mean *Asellus aquaticus* biomass at the start of the feeding trial

The mean biomass of the *A. aquaticus* used in the feeding trial did not differ significantly among the experimental treatments (ANOVA, Table 1). The overall mean in biomass was – 20.48 ± 1.1 per treatment ±SE (standard error). This confirms that the random distribution of the individuals with respect to body size between treatments was successful and well-standardized. There is thus no reason to expect that variation in the start biomass of the individuals used in each treatment would have affected the final result, since each dish contained similarly size individuals capable of consuming the same amount of MP particles. Also as mentioned in the background of this report, the size of *A. aquaticus* individuals may affect their likelihood to survive stressors (Kiffney and Clements, 1996). Having an equal distribution of sizes in the treatments lends to the assumption that size, in this experiment, is an insignificant variable.

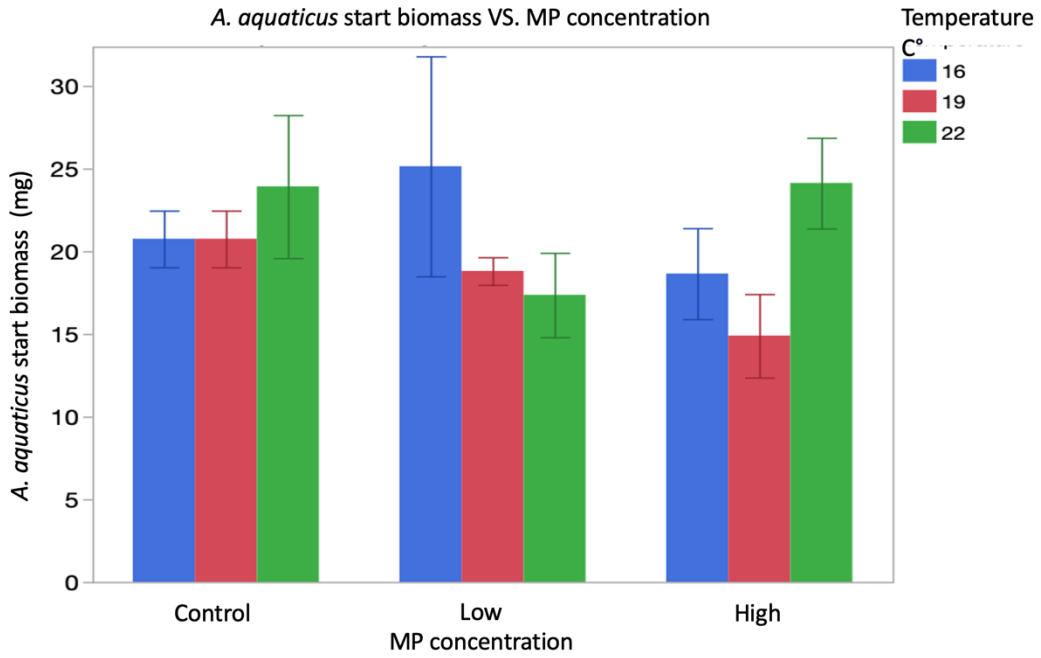

Figure 3

Effect of MP concentration and temperature on mean ±SE biomass of *A.aquaticus* at the start of the feeding trial. The temperatures utilised are shown in blue (16 C°), red (19 C°) and green (22 C°). The control signifies the microcosms in which no plastic particles were added, while low and high indicate the lowest and highest MP concentrations used.

3.2 Mean final *Asellus aquaticus* biomass

The mean final biomass calculated for each treatment differs when compared to the start biomass. The mean final biomass for the *A.aquaticus* was 18.847 ± 0.100 per treatment in the study.

There was no significant effect of any of the experimental treatments on the final *A. aquaticus* biomass (see Table 1 for ANOVA output). This is in line with the lack of any significant differences in survivorship.

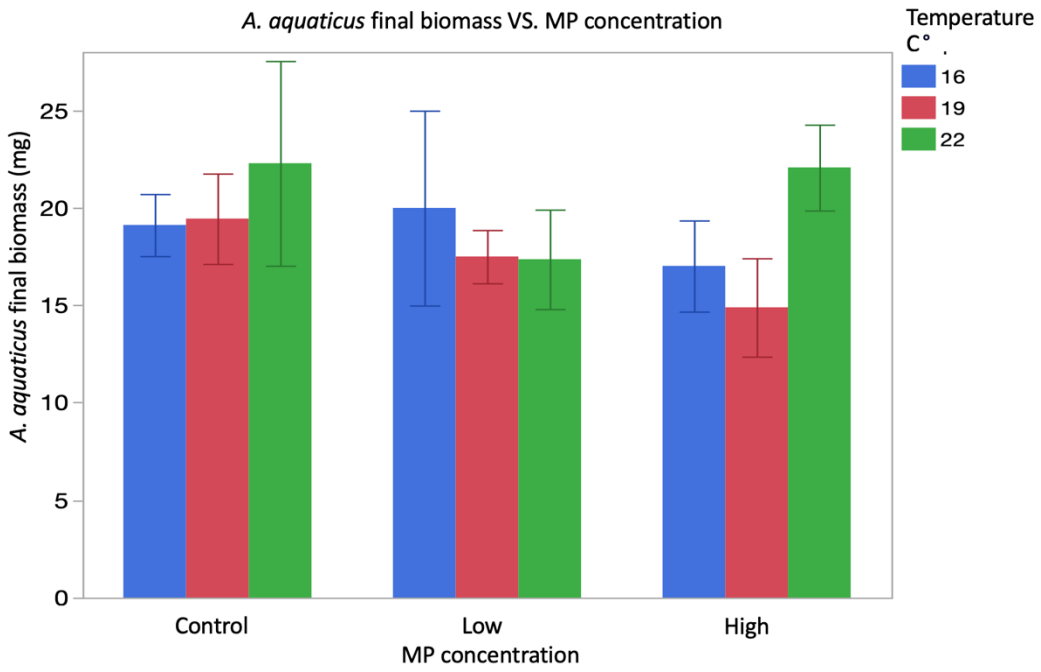

Figure 4
Effect of MP concentration and temperature on mean ±SE final biomass of *A.aquaticus* at the end of the feeding trial.

3.3 Survivorship

Mean *A. aquaticus* survivorship across all microcosms is 92.04 ± 2.37. ±. This shows that the survivorship for all microcosms was relatively high as the mean rate of survival was 92.04%. There was no effect of either temperature, MP concentration of their interaction on survivorship (Table 1).

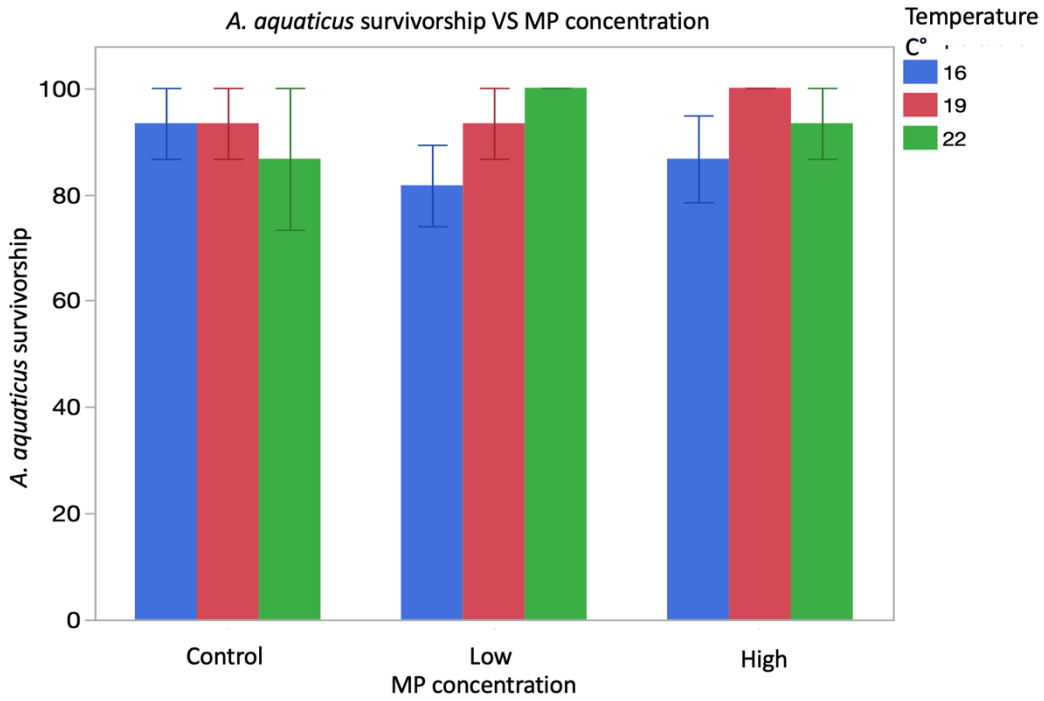

Figure 5

Effects of MPs and temperature on mean ± SE percent survivorship of *A. aquaticus*. Survivorship is measured in percent of the total amount of individuals in each treatment.

3.4 Total microplastic count

MP concentration had a significant effect on the number of particles recorded in the faecal matter of *A. aquaticus* (Table 10). The microcosms containing a higher concentration of MPs also had a higher number of particles found in the faeces of *A. aquaticus*. The sum of the means for all microcosms is 3 ± 0.85. 0.85 being the standard error that was calculated. This shows that the overall ingestion of MPs in all of the treatments and microcosms was relatively low.

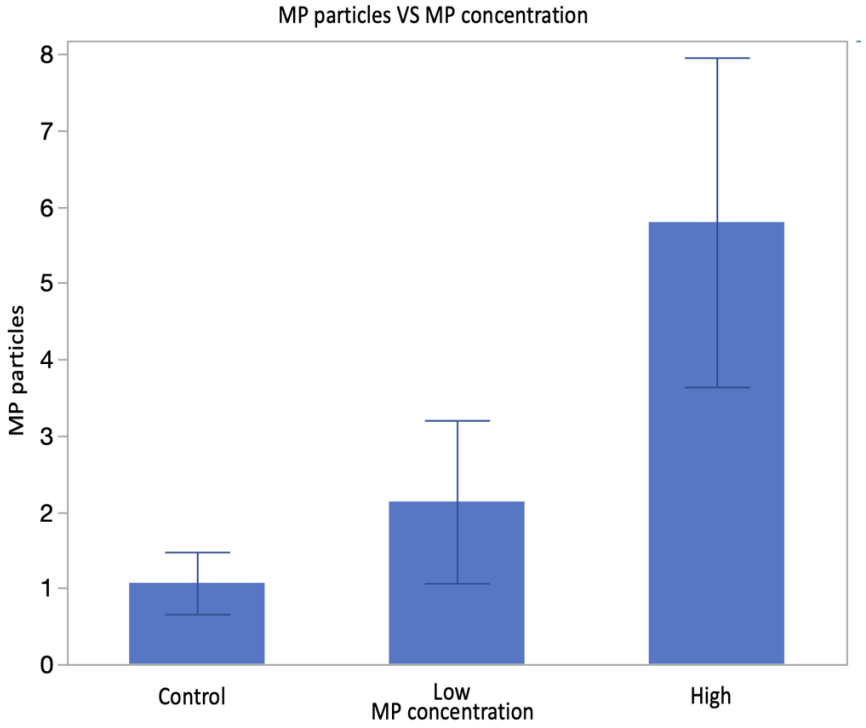

Figure 6
Effects of MP concentration on mean ± SE MP particle counts in *A.aquaticus* faecal particles.

3.5 Controlling for the effects of biomass in particle counts

The overall mean of the particles found per mg of start biomass of *A.aquaticus* is 0.16 ± 0.05 (mean ± Standard Error (SE). The graphs (Fig 5) and table (Table 1) show that the effects of size variation were not driving the result for MP concentration, nor were they obscuring the effect the temperature had on ingestion. Significance tests from the ANOVA are given in Table 1, which also shows all the variables of the effects of biomass in particle counts that were not significant.

The final biomass of the surviving *A. aquaticus* was plotted against the MP concentration in Figure 7 and Table 1. The overall mean after accounting for the loss of biomass is 0.17 ± 0.31(SE). This was done to control if the loss of biomass due to mortality obscured the results. There were, however, no changes in the significance of terms when accounting for the loss of biomass in the ANOVA (Table 1), in other words, it did not factor in the loss of biomass which affects the final results. In a larger study, it could be possible to take a closer look into the

possibility of an increased MP concentration leading to a larger loss of biomass due to mortality.

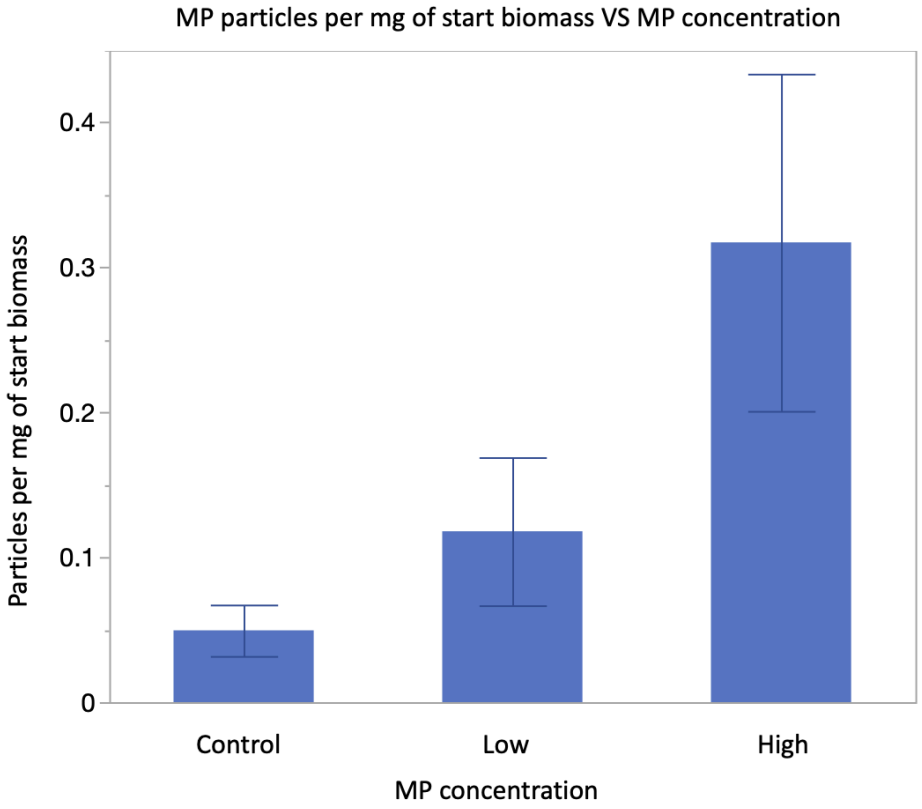

Figure 7
Effects of the MP concentration on the number of particles found per mg (milligram) of start biomass of *A.aquaticus*.

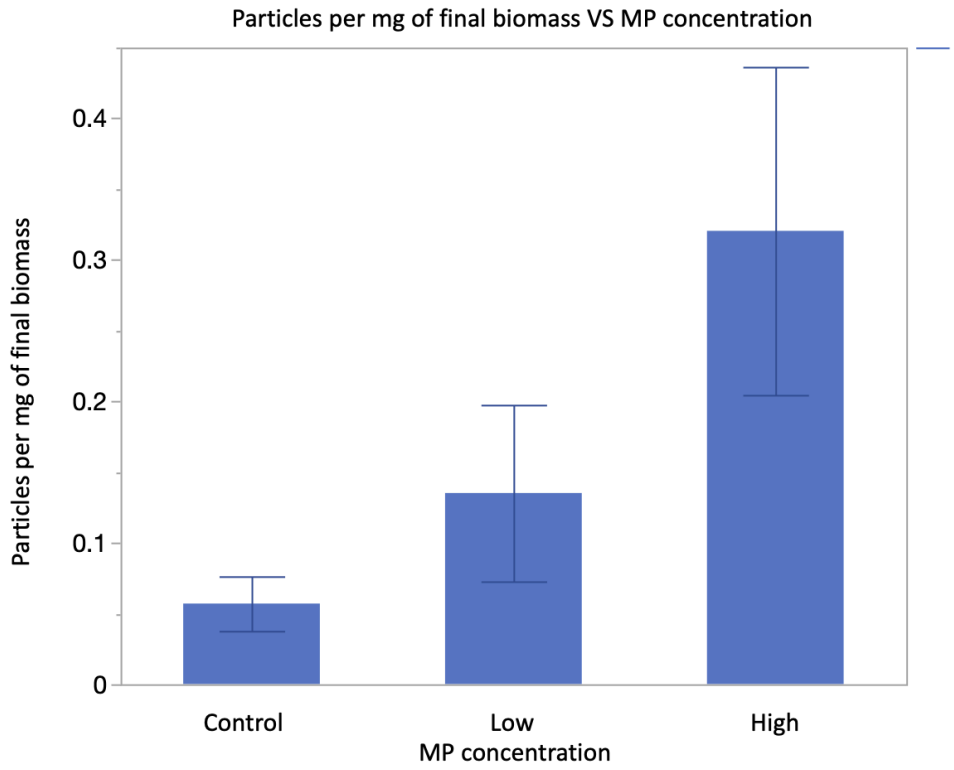

Figure 8
Effect of the number of MP particles found for each mg (milligram) of final biomass of *A.aquaticus* plotted against MP concentration. In order to illustrate if the death of individuals affected the final uptake of MPs in a microcosm.

3.6 *Asellus aquaticus* exuviae

In the graph below the total number of skins counted per treatment are plotted. This shows that more skins were shed when the MPs were present, indicating more environmental stress. This can also be interpreted as *A. aquaticus* attempting to rid themselves of MP contamination by shedding their skin. A similar trend is not apparent for temperature. Unfortunately, it was not possible to conduct an ANOVA analysis on the data for exuviae as this data did not follow a normal distribution and this could not be corrected through data transformation. Nevertheless, these results point towards a hypothesis that could be better assessed in a larger, long term study focusing on the effect of MP concentration on skin shedding in *A. aquaticus.*

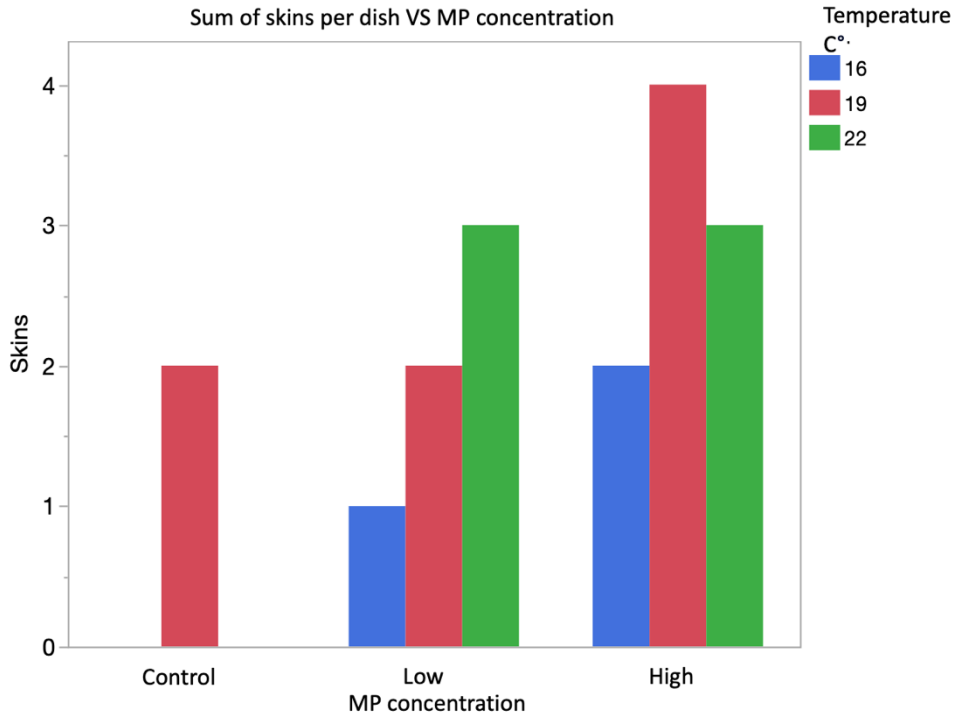

Figure 9
Effects of MP concentration and temperature on the number of skins (exuviae) found.

3.7 ANOVA analysis

Table 1
Output from an ANOVA analysis comparing *A. aquaticus* start biomass, *A.aquaticus* final biomass, survivorship, total MP count, particles per start biomass and particles per final biomass. Tests showing significant results (close to 0.05) are marked with an asterisk.

			A.aquaticus start biomass		A.aquaticus final biomass		A.aquaticus Survivorship		Total MP counts		Particles per mg start biomass		Particles per mg final biomass	
Source	DF	DFDen	F Ratio	Prob > F	F Ratio	Prob > F	F Ratio	Prob > F	F Ratio	Prob > F	F Ratio	Prob > F	F Ratio	Prob > F
Temperature	2	32	0.9485	0.3979	0.5395	0.5882	1.0585	0.3588	0.4872	0.6188	1.0562	0.3596	0.9052	0.4145
MP Concentration	2	32	0.8066	0.4552	0.4074	0.6688	0.0760	0.9270	3.6770	0.0365*	3.8668	0.0313*	3.4892	0.0426
Temperature*MP Concentration	4	32	1.4630	0.2365	0.6098	0.6585	0.8480	0.5054	1.4940	0.2273	1.6540	0.1850	1.7496	0.1635

3.8 Caveats

In this experiment, there was room for a multitude of different faults that could have affected the final results. Most of these are in regard to the human factor together with the risk of cross-contamination between samples, and contamination from other sources specifically regarding the use of the microscope. The most prominent fault in this experiment was the issue with standardisation, as it in some cases became difficult to treat every sample the same. For example, some samples were stained for the recommended 20 mins after vortexing, while others sat in small amounts of dye for days, due to issues with the microscope. Also, the *A. aquaticus* spent different lengths of time outside the temperature-controlled cabinets during the sampling and changing of dishes. The effects this would have had alone on the individuals and ultimately the results are small, but together with other factors could become significant.

Samples not being fully homogenised before analysis proved to be an issue as some large clumps formed in the slides. Despite efforts being made to spread the particles on the side, it was difficult to avoid clumps. These clumps of faecal matter- and potentially MPs, would then light up under the microscope in their entirety, making it impossible to identify MPs within the clump. This led to the possibility of MPs being missed if they were embedded in dense faecal particles. This could have easily been avoided by using for example hydrogen peroxide to dissolve the large faecal particles (as used by Shim et al., 2016). The microscope itself also posed some problems, mostly regarding exposure issues while picture taking. The exposure issues led to the images ranging from high quality (where MPs were visible) to low quality (where the images were overexposed and nothing was eligible). Sadly, this was only noted after the experiment when the opportunity to re-take images had passed. There was also the risk of subconscious bias while taking and analysing the images. To avoid this all images were looked at multiple times throughout the study. Another way to combat this in a future study would be to count the particles in each slide without knowing the treatment that it underwent.

With studies of this nature, there is always the risk of contamination from other sources, especially while working in a non-sterile environment. This can happen despite taking the utmost level of caution throughout the sampling and analysis process. During this study, efforts were taken to reduce the risk of contamination by avoiding plastic containers and tolls for the entirety of the process and by covering the dishes when they were not actively being used. However, airborne MP particles can, in non-sterile environments, come into contact with the samples and ultimately affect the number of particles found. This could then affect the final amount of particles counted in the samples and lead to results that are not

accurate. Contamination or cross-contamination of the samples could be the reason behind control samples (containing no added MPs) having a small number of MPs.

3.9 Conclusions & implications

The main conclusion that can be drawn from this study is that the concentration of MPs in a habitat, or in this case the microcosms, is the most important factor regulating the ingestion and excretion of MPs by *A.aquaticus*. The higher the concentration, the more particles were ingested and seen in the faeces (Figure 6). This is significant and suggests that the *A. aquaticus* lacked the capacity to avoid patches of food resource containing MPs, inevitably also leading to the ingestion of the particles.
In this particular study, the temperature of the microcosms had no impact on uptake (Figure 3). This does not completely rule out that temperature variation can not interact with MP concentration, as it is possible the study was not run for long enough to see a temperature effect develop. Final biomass, also did not differ according to temperature as there was only a very slight difference between the start and final biomass for the *A. aquaticus* (Figures 7 and 8). Had there been a large difference between start and final biomass, indicating death- or growth in a longer study, it would be expected to have a larger impact on bulk ingestion. Thus further supporting the conclusion that the random distribution of individuals in the microcosms was successful (Figure 3).

The results from survivorship (Figure 5) revealed that most individuals survived the study and the mortality was not linked to temperature or concentration of MPs in the microcosm. The presence of MP particles in the faeces can also lead to the conclusion that organisms that then consequently eat those faecal particles will then be further exposed, enabling them to affect others further up the food web (Kong et al., 2023).

This study provides preliminary evidence that MP particles are consumed by *A. aquaticus*, and as a result, occur in their faecal particles. However, more studies focusing on MPs are vital for a greater understanding of their impact, including on organisms subsequently consuming *A. aquaticus* (e.g. fish) or their faecal particles in a wider freshwater food web.

3.10 Future studies

Studies researching MPs and their effects on organisms in different habitats are of great importance as we are only starting to understand their impact. If I had the opportunity to repeat this research, I would run the study for longer to get a clearer test of my hypothesis regarding temperature, MP concentrations, biomass, survivorship and exuviae. A longer trial period would have enabled the individuals to show signs of being affected by the warmer temperatures and the MPs. I would furthermore add more replicates, more individuals and a more diverse array of temperatures. These factors together would increase the possibility of detecting the effects of temperature and MP contamination. Having more replicates in the study could also increase its statistical strength. Allowing stronger and more well-backed conclusions to be drawn.

I used two MP concentrations in the study, representing moderate and extreme levels observed in nature. The use of a greater range of MP concentration and temperature could potentially give clearer results as to if the uptake of MPs varies with temperature. All three temperatures used in this study were much higher than the prevailing temperature at the time I collected *A. aquaticus* from the pond, just after spring ice thaw. As such, all temperatures were likely to have been stressful for the individuals collected.
Another potential aspect for a future study, would be to use temperatures both warmer and colder than the ones used. This would allow us to see how even warmer or colder temperatures affect ingestion. In particular, a greater range of temperatures would allow a better assessment of the ideas shown in Ward & Stanford´s (1982) thermal equilibrium hypothesis, as presented in the introduction. Specifically, whether MP excretion is lowered at both very low and high temperatures, above or below the organism's thermal optimum.

Using higher concentrations of MPs, however, may have limitations as the results from a study where unrealistically high concentrations of MPs are used, may generate results that cannot be applied to natural environments at current levels of MP contamination. Very high concentrations may lead to a strong “food dilution effect”, causing individuals to eat more MPs than they would at lower MP concentrations in a more natural setting, where other food sources are more readily available.
It would also be of interest to analyse more individuals, and species belonging to different biological groups that live in the same ecosystem, in various temperatures and in MP concentrations that could be found in a natural environment.

This approach would be in order to receive an overview of the actual effects that we can expect warmer temperatures and the presence and uptake of MPs to have on organisms across the wider food web.

Another interesting variable that would be of interest to include in a future trial would be to closely analyze the relationship between the number of skins the *A. aquaticus* shed and the MP concentration together with temperature. As mentioned in the background, *A. aquaticus* are proven to shed as a response to stress, including the presence of contaminance. This is a notion that was also proven in the results section of this study, where it was documented that higher MP concentrations showed more frequent shedding. However, a longer study with more replicates and more treatments could shed light on this relationship, potentially leading to an understanding of which MP concentrations and temperatures lead to excessive shedding.

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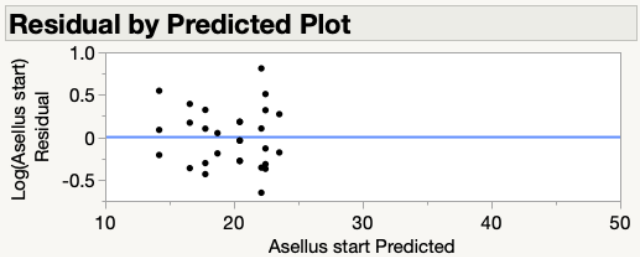
Appendix 1


Residual by Predicted Plot
Log(Asellus start) Residual
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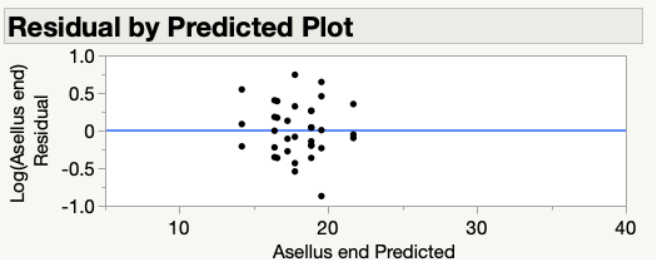
0.5
0
-0.5
10
20
30
40
50
Asellus start Predicted

Appendix 2


Residual by Predicted Plot
Log(Asellus end) Residual
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0
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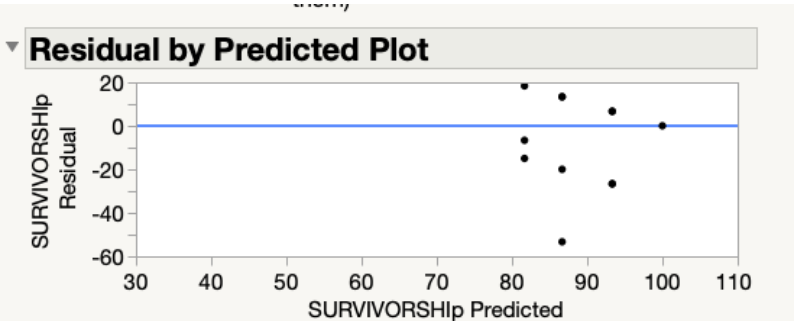
-1.0
10
20
30
40
Asellus end Predicted

Appendix 3


Residual by Predicted Plot
SURVIVORSHIp Residual
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40
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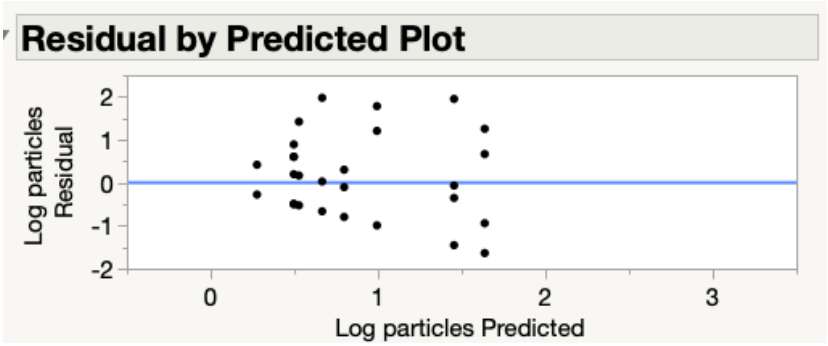
70
80
90
100
110
SURVIVORSHIp Predicted

Appendix 4


Residual by Predicted Plot
Log particles Residual
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1
0
-1
-2
0
1
2
3
Log particles Predicted

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