The effects of microplastic and natural particles on the invasive mussel *Mytilus galloprovincialis* (Lamarck, 1819) and the native *Choromytilus meridionalis* (Krauss, 1848)

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FACULTY OF SCIENCE

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Abstract

Mussels living in coastal environments are often exposed to natural inorganic particles and hence may be well adapted to dealing with high sediment loads. The mechanisms by which they deal with particle loads do, however, cause stress and alter metabolic processes. An increasingly common anthropogenic addition to particle loads in the ocean are microplastic particles. Numerous recent experiments have addressed the impacts of microplastics on metabolic performance, but few of these have used natural reference particles to control for the concurrent effects of particle load itself. This study aims to compare the effects of microplastic and of natural particle exposure on the mussel *Mytilus galloprovincialis*, an invasive species which has become the dominant mussel in the mid- to low-shore of the south and west coasts of South Africa, but is absent from areas prone to sand inundation. These effects will be compared to those on the native mussel *Choromytilus meridionalis*, which resides on the low shore, and unlike *M. galloprovincialis* often occurs in areas prone to sand inundation. Respiration rates, byssus production, clearance rate, body condition (BCI) and survival of mussels exposed to four concentrations of two particle types, polyvinyl chloride (PVC) and red clay were measured. A significant concentration effect was found in the respiration rates of *Mytilus galloprovincialis*, while *C. meridionalis* respiration rates were largely unaffected by both particle type and particle concentration. The byssus numbers of *M. galloprovincialis* were significantly reduced by microplastic exposure, whilst no particle type effects were found in *C. meridionalis*. Clearance rates of *C. meridionalis*, on the other hand, were significantly affected by particle concentration, while no effects were found on *M. galloprovincialis.* The BCI of *C. meridionalis* was also found to be affected by particle concentrations, while *M. galloprovincialis* was unaffected. All *C. meridionalis* individuals survived the experiment, while 29 *M. galloprovincialis* died. Mortality of *M. galloprovincialis* exposed to the two particle types was not significantly different, although more mortality was suffered in PVC treatments than in red clay treatments. The results reveal that there was indeed a difference in the response of *M. galloprovincialis* to the different particle types, and that the two species did exhibit different strategies to both particle type, and concentration. Experimental studies of this nature are imperative in order to disentangle microplastic effects from those of particles in general, and to develop a better understanding of potential impacts of plastic debris on marine ecosystems.

1. Introduction

The accumulation of plastic debris in the ocean is of growing concern both due to its impact on natural ecosystems and on human health (Chae and An 2017, Gabriel et al. 2018, Karbalaei et al. 2018) Since the development of the first synthetic plastic polymer in 1907, plastics have become an integral part of human society, resulting in the rapidly increasing generation of plastic waste. A substantial amount of this waste is transported into the ocean, with an estimated 4.8 - 12.7 million megatons of plastic entering the ocean in 2010 (Jambeck et al. 2015). A large proportion of the plastic debris found in the ocean are microplastics (1µm- 5mm) (Frias and Nash 2019; Hartmann et al., 2019). Microplastic particles either originate from the fragmentation of larger plastic debris due to wave action and sunlight (secondary microplastics), or from their direct use in cosmetics and industrial processes (primary microplastics). Based on a meta-analysis of effect data available in the literature, It has been suggested that the ecological safe level for microplastic concentrations in the ocean is 6650 buoyant particles per cubic meter, beyond which direct effects on a variety of marine taxa become more likely (Everaert et al. 2018). Global microplastic concentrations are not expected to reach this level within the near future, although a few heavily polluted sites across the world already exceed these concentrations, and the numbers of such hotspots are likely to increase in the future. This rapid increase in plastic waste has resulted in a surge of scientific studies assessing the potential impacts of both micro- and macro-plastics on marine biota.

While the effects of macroplastics are well established on a variety of biota (Laist 1997), the effects of microplastics on aquatic life is less clear, despite the rapid increase in publications on the subject. Organisms from a number of functional groups ingest microplastics in laboratory experiments, including filter feeders, deposit feeders and free-swimming predators (Cole et al. 2013, Wright et al. 2013, Neves et al. 2015, Woods et al. 2018). The ingestion of microplastics has also been shown to occur in the natural environment, especially in filter feeding bivalves (Van Cauwenberghe et al. 2015, Li et al. 2016). These include organisms sold for human consumption, with several studies finding consistent numbers of microplastic particles in commercially-sold species, such as mussels, oysters and fish (Li et al. 2015, Bessa et al. 2018). This has raised concerns for human health, although research on the impacts of microplastics on humans has thus far been inconclusive (Karbalaei et al. 2018).

Various physiological effects have also been found on a range of biota due to microplastic exposure. Negative impacts have been documented on feeding, reproduction, growth, development and lifespan within a range of zooplankton taxa (Botterell et al. 2019). Fish have also been shown to be negatively affected, especially in their ability to hunt natural prey in the presence of microplastics (de Sá et al. 2015). In some fish, microplastics have also been found to have adverse effects on growth, reproduction and survival (Foley et al. 2018). A number of studies have shown that deposit feeders are negatively affected, especially lugworms such as *Arenicola marina*, where fitness was negatively correlated with increasing microplastic concentrations (Besseling et al. 2013). Several studies have focussed on mussels, which are filter-feeding benthic bivalves that are very important both ecologically and commercially. Mussels are ecosystem engineers, providing habitat for a wide variety of smaller infaunal species, are involved in nutrient cycling and are also important as the preferred food items for several higher trophic organisms (including humans). Numerous effects of microplastics on mussels have been documented, including reduced attachment strength, lowered filtration and respiration rates and increases in pseudofaeces production and mortality (Rist et al. 2016, Woods et al. 2018, Green et al. 2019). Microplastic particles have also been found to be incorporated into byssus threads, which may play a role in the observed influences of microplastics on byssus production (Li et al. 2019).

Several arguments have been raised questioning the applicability of laboratory experiments on the effects of microplastic particles on marine biota to natural ecosystems (Carlos et al. 2018). Some of the concerns raised include the use of non-ecologically relevant particle concentrations and types, the overuse of spheres instead of more commonly-found fragments and filaments, short experimental exposure times and the low diversity of experimental organisms (Ngoc et al. 2015). Additional concerns are the quality and coverage of microplastic observations in the environment, with a lack of standardization in the techniques and definitions used in monitoring programs (Burns and Boxall, 2018; Hartmann et al., 2019). This has made it difficult to determine what an ecologically relevant microplastic concentration is, and to make accurate predictions about future impacts of microplastics. One of the most integral aspects that has been missing from experimental designs, and which has not been mentioned in critical reviews of microplastic research, is the incorporation of natural reference particles as a control against which to evaluate the impacts of microplastic particles. This omission is somewhat surprising, especially when considering that suspension feeders, which process large volumes of water in order to extract organic particles, are frequently exposed

and even buried by natural sediment (Hutchison et al. 2016), and are often well adapted to survive these conditions.

The filter-feeding process is selective for particle properties such as particle size, type, charge and nutrient content, however the mechanism by which they do so is poorly understood (Rosa et al. 2013). At low particle concentrations mussels filter particles larger than a few microns in diameter from the water using their gills, gather these in mucus strings and transport them to the mouth where they are ingested (Kiørboe and Møhlenberg 1981). Not all these ingested particles enter the digestive glands and as particle suspensions get too dense, the mussels become increasingly selective and may sort and digest the lighter organic particles, while allowing heavier sedimentary particles to pass thought the gut without entering the digestive glands. At very high particle concentrations the digestive system becomes overwhelmed and the strings of mucus in which filtered microparticles are carried towards the mouth are rejected in their entirety, and expelled through the exhalent aperture in the form of pseudofeces (Foster-Smith 1975). Most experimental evidence is consistent with the suggestion by Winter (1977) of a three-phased response by bivalves to increasing particle loads (Griffiths and Griffiths 1987). The first response is to rapidly increase the pumping rate after the breaching of a fairly low particle concentration threshold. The second phase occurs over what are likely to be optimal feeding concentrations and is characterized by a plateau in the pumping rate. At particle concentrations above this phase, pumping rates are consistently slow, and pseudofeces production rapidly increases. The process by which pseudofeces are produced is energetically demanding and reduces the efficiency of the filter feeding and energy acquisition process but is an effective measure for coping with high sediment loads. Before the threshold at which psuedofeces production commences, mussels are more susceptible to consuming particles such as microplastics and inorganic sediment (Kiørboe and Møhlenberg 1981). High inorganic particle loads therefore act to dilute the organic portion of suspensions, thereby decreasing the energy content of the filtrate, resulting in metabolic stress. For example, Roper and Hickey (1995) found that respiration and filtration rates in the freshwater mussel *Echyridella menziesii* increased with increasing silt concentrations when food concentrations were low, showing that high inorganic particle loads with low organic particle concentrations results in mussels having to increase metabolic activity to obtain sufficient amounts of organic materials. Mussels are, however, well adapted to surviving with this trade-off and proliferate in coastal zones that are very prone to high sediment loads.

Inorganic particles can also cause more direct impacts on filter feeders. When particle concentrations are high, the filtering apparatus may clog, which reduces the efficiency of the filter-feeding process (Rubenstein and Koehl 1977). This has also been shown to occur in Cladocera, where clay mechanically interferes with the collection and ingestion of organic particles (Kirk 1991). Ellis (1936) noted that dying mussels had accumulations of silt in the gills and mantle cavity and that mussels were often observed to close their valves in the presence of high particle concentrations. The blocking of feeding appendages has also been proposed to occur with microplastic particles, but whether or not this occurs more frequently with the latter than with natural particles is yet to be established (Derraik, 2002). Additionally, both microplastics and natural sediment have been found to be carriers of toxins, such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls (PCBs) in the environment (Sapozhnikova et al. 2004, Rios Mendoza and Jones 2015). Microplastic particles, especially smaller particles including nanoplastics (1-100 nm), can translocate into the circulatory systems of mussels and hence potentially leach harmful chemicals (Browne et al. 2008, Sendra et al. 2019), but it has not yet been established whether or not this process occurs also with natural particles.

In light of the well-established physiological changes induced by increasing concentrations of natural microparticles on mussels and the lack of convincing evidence that microplastics behave very differently to natural particles in the natural environment, it is a major shortcoming that the overwhelming majority of studies on the impacts of microplastic have not included the effects of natural particles as a control in their experimental designs. This makes it impossible to determine whether effects found can be attributed to the inherent properties of the plastic particles themselves rather than to increasing particle concentration in general. Whilst seemingly obvious, this shortcoming has not been highlighted in critical reviews on microplastic research (Cole et al. 2011, Carlos et al. 2018).

A few recent studies have focussed on disentangling the effects of natural and microplastic particles. Harris and Carrington (2020) compared the effects of silt and microplastics on the clearance rates of *Mytilus trossulus* (Gould, 1850) and showed that microplastics led to a reduction in clearance rates by 62% at the highest concentrations, but this did not occur at similar silt concentrations. Despite the fact that mussels have been documented to reduce their clearance rates in the presence of natural particles (Ali 1970), this is a clear indication that mussels may interact with the two particle types differently. Effects of microplastics when compared to natural particles are also apparent in *Daphnia magna* (Straus, 1820), with reduced

reproduction and feeding at microplastic concentrations >10000 particles per millilitre, but no effects being found at equivalent kaolin concentrations (Ogonowski et al. 2016). There is thus some evidence to suggest that natural and microplastic particles differ in their effects, and microplastics could be a novel stressor in the marine environment. Further studies of this nature are, however, required to better understand these differences, and to develop a more complete understanding of the drivers of differential effects of microplastics and natural particles. Such findings can give greater insights into potential ecological impacts such as effects on energy transfer, benthic-pelagic coupling and food web interactions which have been alluded to in the absence of such comparisons (Galloway et al. 2017).

This study aims to compare and contrast the effects of microplastics and natural inorganic particles at similar concentrations on mussels. To conduct this comparison, a series of laboratory experiments were done using *Mytilus galloprovincialis* and *Choromytilus meridionalis* (F. Krauss, 1848) as model organisms. *Mytilus galloprovincialis* is closely related to *M. edulis* (Linnaeus, 1758), which has been the model organism for the majority of the experimental research on mussels. These two species have been shown to hybridize, and their relatedness ensures that the results from this experiment could be easily compared to the wealth of literature on *M. edulis* (Skibinski et al. 1978). *Mytilus galloprovincialis* is a common and commercially important species in Europe and was accidentally introduced to South Africa in the mid-1970s, where it has since become widespread along the entire west coast and (at lower abundances) also the south coast. Beds of *M. galloprovincialis* are mostly confined to the intertidal zone where they house a very similar infaunal community to indigenous mussel species, such as *C. meriodionalis* and *Perna perna* (Linnaeus, 1758; Hammond and Griffiths, 2004). They do, however, have significant impacts on various native fauna, with some dramatic examples already documented (Branch et al., 2004; Robinson et al., 2007). *Mytilus galloprovincialis* has been found to outcompete slower-growing indigenous mussel species such as *Aulacomya atra* (Mörch, 1853), and its success has largely been attributed to its greater reproductive output, faster growth rate and better tolerance to desiccation (Hockey and Van Erkom Schurink, 1992). However, *M. galloprovincialis*, has not been found to significantly affect either *P. perna* or *C. meridionalis*, largely due to partial habitat segregation (Bownes and McQuaid, 2006). *Choromytilus meridionalis* is of particular interest when considering the question of tolerance to inorganic particles, as it has been proposed by a number of authors that it is able to survive buried in sand, and thus rarely overlaps with *M. galloprovinvialis*, as the latter is less tolerant to sand stress (Bownes and McQuaid, 2006; Marshall and

McQuaid, 1993; Zardi et al., 2006). This study will therefore investigate whether exposure to inorganic particles, both microplastic and natural, will have contrasting effects on *C. meridionalis* and *M. galloprovincialis*, as the two species occupy very different habitats. Such comparisons are important to better understand responses towards stressors such as suspended particles and may be useful in making more accurate predictions about the potential of *M. galloprovincialis* to further expand its distribution range and consequent impact on native fauna and ecosystems (Branch et al. 2004).

Following the Introduction and Methods chapters, the third chapter of this dissertation presents the Results and Discussion of Experiment 1 in which I compared the effects of microplastics and natural inorganic particles on respiration rate, byssus production, filtration rate, Body Condition Index (BCI) and mortality of *M. galloprovincialis.* For Experiment 1, I hypothesized, (i) that exposure to microplastic particles will result in reduced health in *M. galloprovincialis*, and (ii) that increasing particle concentrations will negatively affect the above-mentioned physiological parameters. In the fourth chapter of this dissertation, I present the Results and Discussion of Experiment 2, in which I compared the effects of the two particle types and concentrations on respiration rate, byssus production, BCI and mortality of *M. galloprovincialis* and *C. meridionalis*. For Experiment 2, I hypothesized that, (iii) *M. galloprovincialis* and *C. meridionalis* will differ in their responses to particle type, and (iv) that increasing concentrations of suspended sediments will have greater impacts on *M. galloprovincialis* than on *C. meridionalis*.

2. Methods

The methods outlined in this chapter apply to both of the two following experiments, for which Results and Discussions are provided in sections 3 and 4, respectively. Experiment 1 focuses on comparing the response of the invasive mussel *Mytilus galloprovincialis* to exposure to microplastics versus natural clay particles, while Experiment 2 compares the responses of this mussel with that of the native *Choromytilus meridionalis*. The response variables measured are the same for both experiments, and thus a combined Methods section is provided in this section.

2.1. Experimental design

For Experiment 1, a total of 90 individuals of *M. galloprovincialis* (shell length = 2.70 ± 0.150 cm) and for Experiment 2 an additional 90 individuals of *C. meridionalis* (shell length = 2.73 ± 0.146 cm) were exposed to four different concentration levels of microplastic and of natural particles for a total exposure time of 58 days between July-September (Figure 1). Respiration rates, filtration rates and byssus numbers were measured after three and six weeks of exposure. Dry weights were measured in week seven to calculate the Body Condition Index (BCI). For the respiration rate, filtration rate and byssus number measurements, individuals were placed in containers with sea water filtered to 1 µm in the absence of particles. The measurements were therefore aimed at measuring 'carry-over effects' following prolonged exposure to particular particle concentrations, rather than direct effects in the presence of particles during measurement. Before assigning individual mussels to the different treatment levels, baseline measurements of all variables, except filtration rate, were taken to ensure that there were no significant differences between the groups before the experiments began. These baseline measurements were also used to compare respiration rates and byssus production of *M. galloprovincialis* and *C. meridionalis* before being exposed to the experimental particles. An additional 24 *M. galloprovincialis* (shell length = 2.68 ± 0.13 cm) and 20 *C. meridionalis* (shell length = 2.76 ± 0.17 cm) individuals, from the same sites where the experimental mussels were collected, were used to assess the BCI of the two species before the start of the experiment.

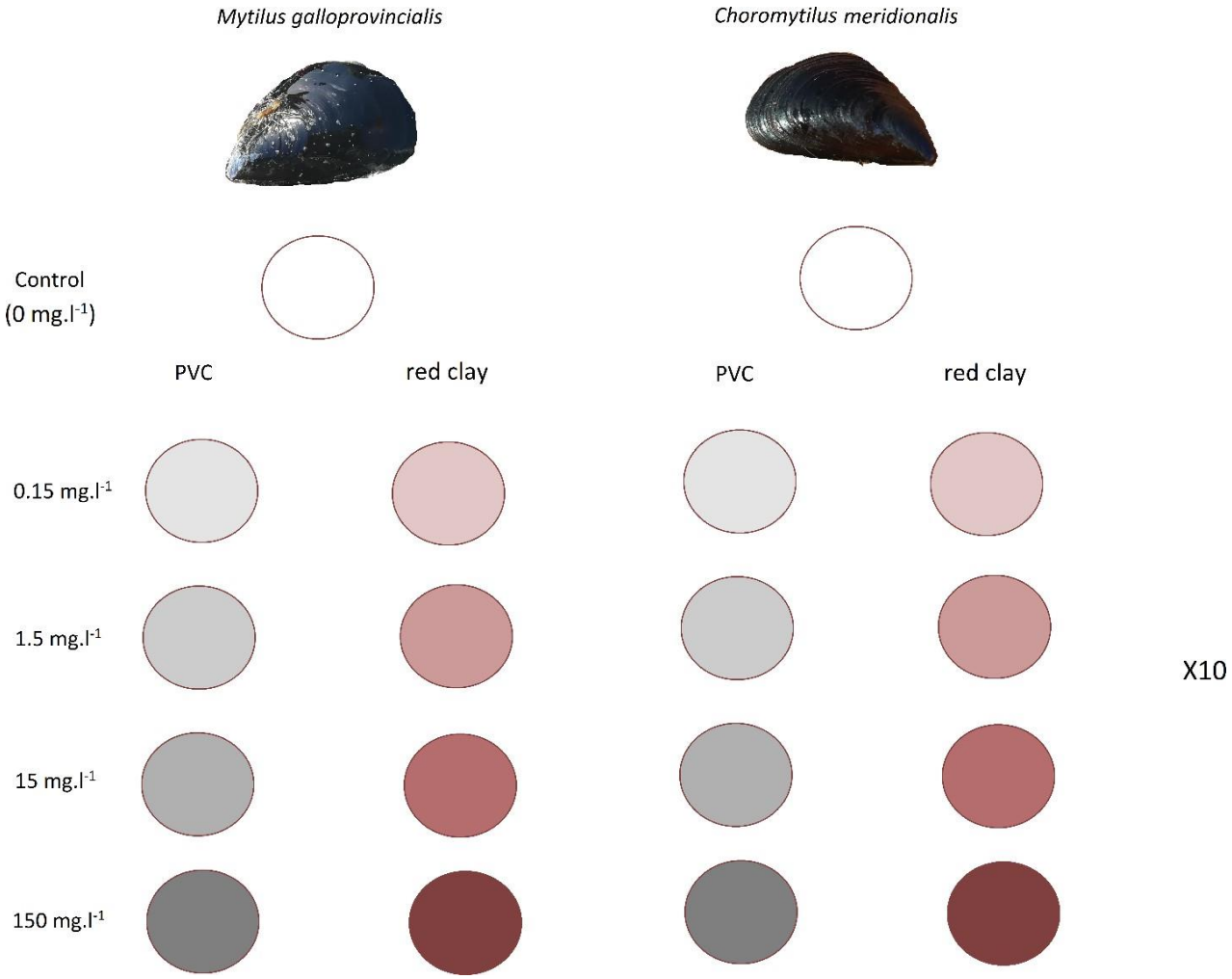

Figure 1. Schematic of experimental design. For each of the two species of mussels, two different particle types, i.e. PCV (grey) and red clay (red), were compared at four different particle concentrations (shadings), and a control treatment with no particles included. Each treatment combination was replicated ten-fold.

2.2. Collection and maintenance of test organisms

Individuals were fed daily with *Pavlova lutheri* (4-6 µm), a unicellular marine haptophycean flagellate, to achieve an algal concentration of 15 000 cells l^{-1} in the experimental units. Algae starter cultures were provided by the Department of Environment, Forestry and Fisheries (DEFF) research aquarium and were grown in the Centre for Bioprocess Engineering Research (CeBER) in the Department of Chemical Engineering, University of Cape Town. Algae were cultured in Erlenmeyer flasks with artificial sea water and nourished with Walne's medium. Before feeding, algal cells were counted under a microscope using a Neubauer Improved® counting chamber and the amount fed was adjusted according to the algae culture concentration. Algae were deposited into the experimental units with a multistep pipette.

2.3. Experimental setup

The experimental components of the project took place at the Department of Biological Sciences, University of Cape Town, South Africa. The two experiments were carried out in a climate chamber under stable environmental conditions. The temperature was maintained at 13.5-14.5°C and the lights set to a 12-hourly day/night cycle. Sea water filtered to 0.2 µm was collected regularly from the Department of Environment, Forestry and Fisheries (DEFF) research aquarium in Sea Point, Cape Town, and stored in 1000 l tanks in the climate chamber for use throughout the experiment. The water was filtered again through a series of 100 µm, 10 µm and 1 µm filters prior to use. Salinity levels were measured weekly and always fell in the range 35-37 psu. Nitrite, nitrate, phosphate and pH levels were also monitored weekly with a water quality testing kit to ensure water quality levels were optimal throughout the exposure time and these never reached harmful levels.

The experimental setup is shown in Figure 2. Experimental units consisted of 1.5 litre repurposed plastic soft- drink bottles. After cutting off the bottoms, the bottles were placed upside-down in crates, with the aeration pipe extending from the top, down to the lid at the bottom to ensure efficient resuspension of particles and to minimize accumulation of particles in the lid. One mussel was placed in each experimental unit. The positions of the crates in the climate chamber were rotated weekly, as were the positions of the bottles in the crates, to reduce the influence of any variability in environmental conditions within the climate chamber. For each of the two experiments, the experimental design consisted of 10 replicates of four concentration levels of microplastic and of natural particles; a control group consisting of mussels in a particle-free environment was also included, resulting in a total of 90 mussels. A full water change, including cleaning the bottles was done daily , except for Sundays. Each bottle was filled with 1 litre of water and aerated continuously for the entire experiment.

Figure 2. Part of the experimental setup, showing the aeration system, crates and experimental units (left half), and close up of one of the crates with bottles containing the 150 mg.l^{-1} concentrations of PVC (left) and red clay (right) (right half). Photographs by Silja Blechschmidt.

2.4. Preparation of treatment levels

The particle types used were red montmorillonite (red clay) with a median size of 12.08 µm as the natural reference, and polyvinyl chloride (PVC) with a median size of 13.84 µm as the microplastic. The PVC powder was purchased from PyroPowders® and the red clay from Now Foods®. Both powders were free of additives and were irregularly-shaped to better represent what is found in the environment and had a similar particlesize distribution (Figure 3). The size distributions were also similar to those found in natural suspensions in the marine environment (Puls et al. 1997). Fragmented PVC is an environmentally relevant choice, as it forms a considerable proportion of the plastic waste in the ocean (Auta et al. 2017). Red clay was used as the natural particle as clays are widely distributed throughout the world's oceans and have been documented in considerable concentrations in the south Pacific (Peterson and Griffin 1965, Griffin et al. 1968).

The concentration levels for the two particle types used were: 0.15, 1.5, 15 and 150 mg L^{-1}. These concentrations were chosen to correspond to a range of observed natural sediment concentrations. The highest two concentrations do occur in the ocean, especially in environments with high sediment loads due to river outputs and coastal erosion (Milliman and Meade 1983), and are certainly attainable microplastic concentrations in the future, given the current rate of plastic waste accumulation in the ocean. Additionally, a reference group without particles was included as a control. The four concentration levels used were created by diluting stock suspensions, which were created weekly. The stock suspensions were made with distilled water with the addition of a small amount of biologically-inert surfactant (0.01% Tween 20 solution), which allows particles to mix more homogenously and prevents formation of aggregates. Surfactants such as Tween 20 have been shown to have minor effects on microbes (Yeh et al. 1998), but have been widely used in biological experiments and are considered non-harmful. All concentrations and the control group were treated with the surfactant. The stock suspensions were created so that 10 ml would be added to each unit in order to achieve the desired concentration levels. Wastewater was filtered through a series of 100, 10 and a 1 µm filters to ensure particles were removed prior to disposal.

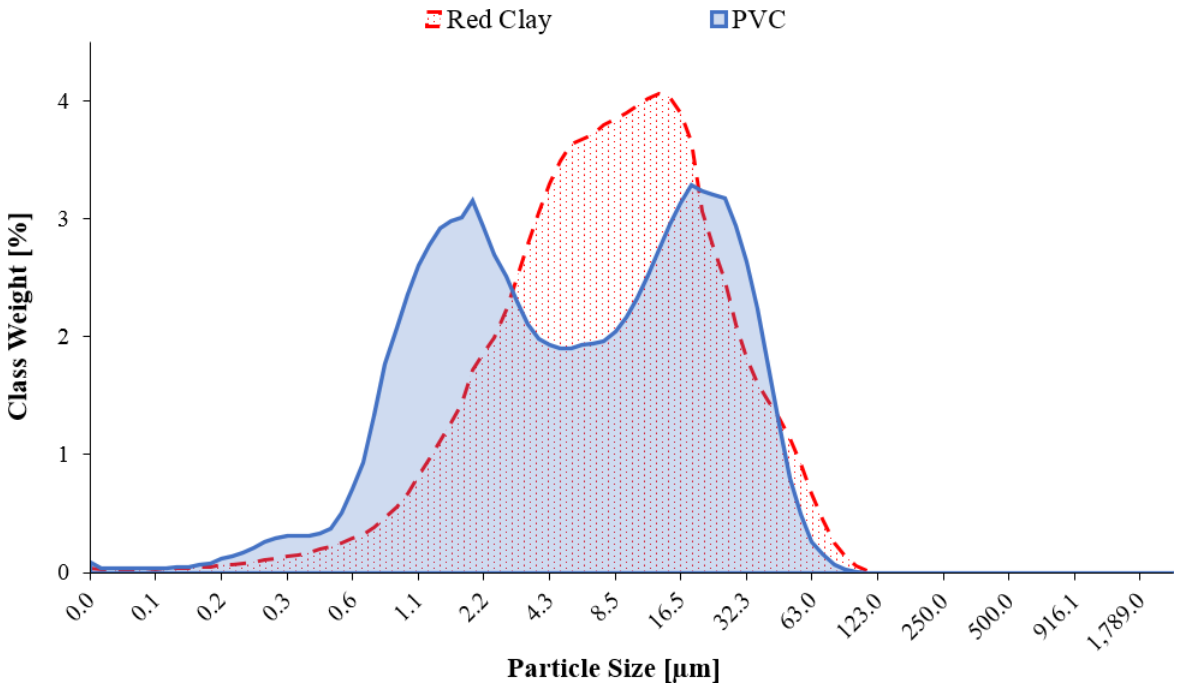

Figure 3. Size distribution of PVC (Mean: 12.08 µm) and red clay (Mean: 13.84 µm) particles used in this study; data were obtained from the analysis of 1 g each powder using CILAS® 1180 laser particle sizer (data and Figure from: Barkhau and Gottschalck, unpublished).

2.5. Respiration rate

Respiration rates were calculated by measuring the oxygen consumption over a fixed time-frame, as described in (Lampert 1984). To measure respiration rates, a large bucket of well-aerated water was prepared, and its oxygen concentration carefully monitored using a WTW Oxi 3315 with an optical probe. Individual mussels were placed in well-sealed glass containers 145 ml in volume. The containers were filled by submerging them in the well-aerated bucket, taking great care that there were no air-bubbles, and then closing them while still submerged. The oxygen concentration was recorded immediately before the jar was sealed, giving C_i. Individuals were left in the jars to respire for 45 minutes, after which the jar was quickly opened and an oxygen probe inserted in the water (C_m). During the two minutes required to take the measurement, the jars were placed on a magnetic stirrer to homogenize the water. The measurements were done in batches of 10, and for each batch a control measurement (with no organism) was included (C_c). Respiration rates were calculated using the following equation from Lampert (1984).

$$Respiration\ rate\ (mg\ O_2 h^{-1}.g^{-1}) = \frac{(\frac{C_i - C_m}{t_m} - \frac{C_i - C_c}{t_c}) \times \frac{V}{1000}}{WW}$$

Where:

C_i = Initial oxygen concentration ($mg.l^{-1}$)

C_m= Oxygen concentration after time (t) with mussel ($mg.l^{-1}$) C_c= Oxygen concentration after time (t) without mussel ($mg.l^{-1}$) t_m= Time of incubation with mussel (h)

t_c= Time of incubation without mussel (h) V= Volume (l)

WW =Wet weight of mussel (g)

2.6. Byssus number

To measure rates of byssal thread production, individuals were placed in glass vials in their experimental units for 24 h, after which the numbers of attached byssus threads were counted. The measurements were done in the absence of particles, so before the 24-h period, the experimental units were carefully cleaned. Great care was taken to ensure that the counted threads were indeed attached, those that were not attached being excluded from the analysis.

2.7. Body Condition Index (BCI)

Assessing the body condition index (BCI) required sacrificing the experimental mussels, so could only be measured at the end of the experiments. The soft tissue was carefully separated from the shell and both dried in a drying oven for 48 h at 60°C. Prior trials were conducted to ensure that this period and temperature were sufficient to achieve constant mass. The mass of the tissue and shell were then measured using a high precision scale. The BCI was calculated using the following equation, which is the best index to represent the adult static condition of bivalves (Lucas and Beninger 1985).

$$(2)\ BCI\ (g.cm^{-1}) = \frac{Dry\ Weight(g)}{Shell\ length(cm)}$$

2.8. Clearance rate

Clearance rate is defined as the volume of water filtered per unit time, and can be calculated by measuring the reduction in suspended material due to filtering over a defined period (Coughlan 1969). For this measurement, individual mussels were placed in 200 ml of 1 micron filtered sea water and allowed to acclimatize for 30 minutes. The mussels were starved for 24 h prior to start of the experiment. After the acclimation phase, algae cultures were counted and added to the bottles to achieve a cell density of 50 000 cells.ml^{-1}. Immediately after supplying algae to an experimental unit, the water was thoroughly stirred and a sample taken with a 5 ml Eppendorf tube. Mussels were left to filter for 45 min, after which another sample was taken following the same procedure. A Becton Dickenson (BD) LSR II flow cytometer with a Blue (75mW), Red (40mW), Violet (75mW) and Green laser (100mW) configuration was used to count the algal cells and only mussels that were open prior to taking the second algal sample were included in filtration rate measurements. All samples were preserved using Lugol's solution and stored at -80 °C until they could be processed by the flow cytometer. At week six of the experiment, the number of replicates in some treatments was as low as two, since most individuals were closed during the measurement. Thus, only data after four weeks of exposure were included in the analyses.

Clearance rates were calculated using the following formula from Coughlan, 1969 (3).

(3) $$Clearance\ rate\ (l\ h^{-1}g^{-1}) = \frac{\frac{V}{t}\times \ln\frac{C_0}{C_t}}{WW}$$

V = Volume of water

t = time

C_0 = Algal concentration at t_0

C_t = Algal concentration at t_1

WW = Wet weight (g)

2.9. Survival

Mussel condition was assessed every day before every water change. Widely gaping individuals were lightly tapped to see if the valves shut in response to stimuli. If the valves did not close, the tissue was carefully examined for any movement and to evaluate whether the mussel was still alive. If the tissue was visibly degraded and/or had no sign of movement, the individual was classified as dead.

2.10. Data analyses

Data analyses were done using the computing software R (version 3.5.3, R Core Team, 2017). All graphs were made using the ggplot2 package (Wickham, 2016). Prior to performing the analyses, all variables were tested for the conditions of normality and equal variance required by parametric statistics. The model assumption of normality of residuals was evaluated using histograms and Shapiro-Wilk's test, while homogeneity of variance was assessed by plotting residuals against fitted values. The presence of influential data points was tested using Cook's distance, and if found, these data points were omitted. Influential data points were only found in the BCI values of *C. meridionalis*, with three points found and subsequently removed.

The level of significance was set at $\alpha<0.05$, however, when p-values fell between $0.05 \leq \alpha \leq 0.10$, results were considered as marginally significant.

Mussels that were closed during respiration and clearance rate measurements were excluded from the analyses. Furthermore, clearance rate values of ≤ 0 recorded with open mussels were also excluded. Size corrections for clearance rate and respiration rates were done by dividing the measured values by the wet weight of the individual. For byssus production, only mussels that were attached to the vials were considered, hence mussels that failed to produce byssus were excluded.

For Experiment 1, the effects of particle type, concentration, and their interaction on the respiration rate and BCI of *M. galloprovincialis* were evaluated using a two-way analysis of variance (ANOVA). A factorial design was used with particle type and concentration as fixed factors. Since ANOVA assumptions did not hold for byssus production and clearance rate, generalized linear modelling (GLM) with a quasi-Poisson link-function

was used to test the influence of the same fixed factors and their possible interactions. Tukey HSD *post-hoc* test was used to compare pairwise differences between treatment combinations.

A survival analysis was conducted in Experiment 1 using the survival package in R (Therneau 2020) to assess whether mortality differed between particle types and concentrations in *M. galloprovincialis*. To simplify the analysis of survival at different concentrations, the two highest and lowest concentrations were pooled and compared. Kaplan-Meier curves were used to visualize mortality events over time. Cox proportional hazard models were used to test whether there were any differences between the survival curves of mussels that were exposed to the two particle types and two concentration groups. The model assumption of proportional hazards was tested by testing the independence between Schoenfeld residuals and time. Dfbeta values were assessed to ensure there were no influential data points.

For Experiment 2, to test whether the respiration rates and body condition indices (BCI) of *M. galloprovincialis* and *C. meridionalis* responded differently to particle type and concentration levels, a three-way ANOVA was done with species, particle type and concentration as the three fixed factors. Since byssus number and clearance rate did not meet the assumptions of ANOVA, GLM was used to test the same factors for these two variables. The interaction between these factors was also tested for both ANOVA and GLM scenarios. Pairwise differences between treatment levels were analysed using Tukey's HSD (honestly significant difference) *post-hoc* tests.

Choromytilus meridionalis suffered no mortality, and hence a survival analysis was not done for Experiment 2.

To assess whether baseline measurements differed between the two species, two-sample t-tests were done for respiration rates and BCI, and Wilcoxon's test was used to test byssus numbers as their distribution did not meet parametric assumptions.

3. Experiment 1: The effects of microplastics on the physiology of the invasive mussel *Mytilus galloprovincialis* (Lamarck, 1819) using natural sediment as a reference.

The Results and Discussion outlined in this chapter build upon the Introduction and Methods described above.

3.1. Results

Respiration rate

After three weeks of exposure, no significant effects on the respiration rates were found in mussels exposed to different particle types, or concentrations, nor were there any significant interactions between the two factors (Table 1). Median respiration rates of all mussels, regardless of to which concentration levels and particle types they were exposed, fell within the interquartile range of the control group (Figure 4a).

However, after six weeks of particle exposure, a significant effect of concentration was found on mussel respiration rates (Table 1). *Post-hoc* tests revealed that this concentration effect was driven by differences between respiration rates in the lowest and highest concentrations, and a general increase in respiration rates with concentration level can be seen ($p = 0.047$) (Figure 4b). The highest respiration rates were recorded in the 150 $mg.l^{-1}$ PVC treatment (Figure 4b). Respiration rates nearly halved between three and six weeks of exposure, with mean (± SD) respiration rates of 0.021 ± 0.008 mg O_2 $.h^{-1}.g^{-1}(ww)^{-1}$ ($n = 78$) after three weeks of exposure and 0.011 ± 0.006 mg O_2 $.h^{-1}.g^{-1}(ww)^{-1}$ ($n = 67$) after six weeks of exposure, indicating a general decrease in mussel vigour. The medians of the 1.5 $mg.l^{-1}$ PVC group, 15 $mg.l^{-1}$ red clay group, and 150 $mg.l^{-1}$ PVC group fell within the interquartile range of the control group, whilst all other treatment levels fell below this (Figure 4b).

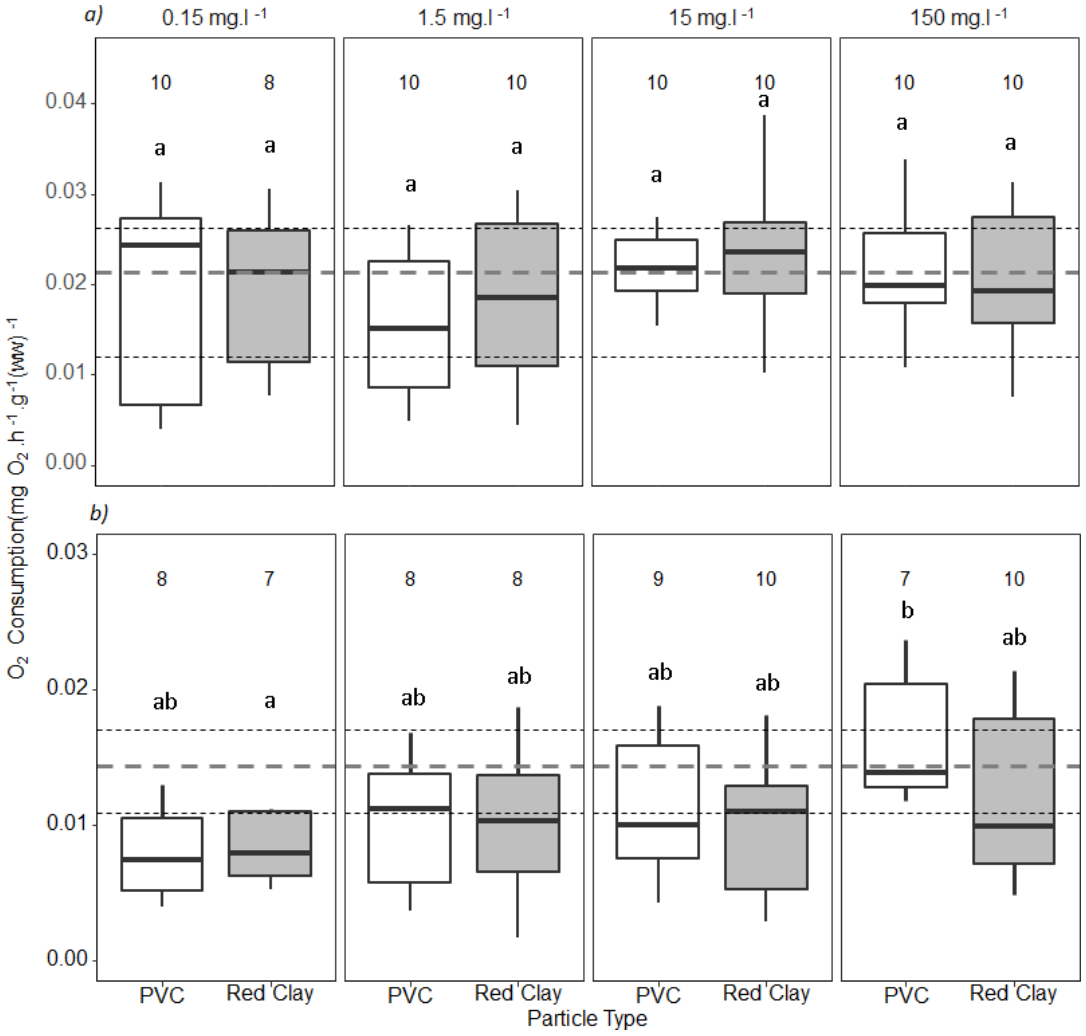

Figure 4. *Mytilus galloprovincialis* respiration rates of individuals after a) three and b) six weeks of particle exposure for each particle type and concentration level. Thick grey dashed line = Median respiration rate of control group (group without particle exposure), thin black lines= lower and upper quartile of control. The number above each bar indicates the number of replicates (N). *Post-hoc* test results are represented by letters above bars, with significant differences indicated by different letters.

Table 1. Summary of two-way ANOVA results of the effect of particle type, concentration and the interaction of the two factors on the respiration rates and BCI of *Mytilus galloprovincialis*.

	Sum Sq	Df	F	P
Respiration Week 3				
Particle type	0.00002	1	0.288	0.594
Concentration	0.00029	3	1.381	0.256
Particle type*Concentration	0.00007	3	0.317	0.813
Respiration Week 6				
Particle type	0.00004	1	1.339	0.252
Concentration	0.00026	3	3.027	0.036*
Particle type*Concentration	0.00005	3	0.533	0.662
BCI Week 6				
Particle type	0.00142	1	1.076	0.306
Concentration	0.00446	3	1.130	0.348
Particle type*Concentration	0.01440	3	3.644	0.021*

Table 2. Summary of GLM results of the effect of particle type, concentration and their interaction on byssus number and clearance rates of *Mytilus galloprovincialis*.

	Chisq	Df	P
Byssus number Week 3			
Particle type	3.1152	1	0.078
Concentration	12.0794	3	0.007**
Particle type*Concentration	3.8294	3	0.280
Byssus number Week 6			
Particle type	5.8567	1	0.016*
Concentration	2.2365	3	0.525
Particle type*Concentration	1.9295	3	0.587
Clearance rate Week 3			
Particle type	0.85005	1	0.357
Concentration	2.17058	3	0.538
Particle type*Concentration	1.92634	3	0.588

Byssus number

After three weeks of particle exposure, a marginally significant effect of particle type, and a significant concentration effect was observed on the byssus production (Table 2). Mean ± SD byssus numbers after PVC exposure was 7.41 ± 4.80 threads per individual, while mussels exposed to red clay had mean byssus numbers of 9.50 ± 6.12 (see Appendix Figure 18). *Post-hoc* testing found that byssus production differed significantly between the 0.15 $mg.l^{-1}$ and 15 $mg.l^{-1}$ ($p = 0.027$), and between 15 $mg.l^{-1}$ and 150 $mg.l^{-1}$ concentration levels ($p = 0.018$) (Figure 5a). The median byssus number produced in most of the treatments fell within the interquartile range of the control group (Figure 5a), except for the lowest PVC concentration, and second highest red clay concentration.

After six weeks of exposure mussels exposed to PVC had significantly lower byssus production than those exposed to red clay (Table 2). Mussels exposed to PVC produced a mean ± SD of 3.72 ± 4.23 byssus threads per individual, whereas mussels exposed to RC produced a mean ± SD of 5.77± 7.44 byssus threads (see Appendix Figure 15). This difference becomes apparent only at the two highest concentrations (Figure 5b). It is important to note that, except for the lowest concentration, the median number of byssus threads in the mussels exposed to red clay was higher than the median and interquartile range of the control (Figure 5).

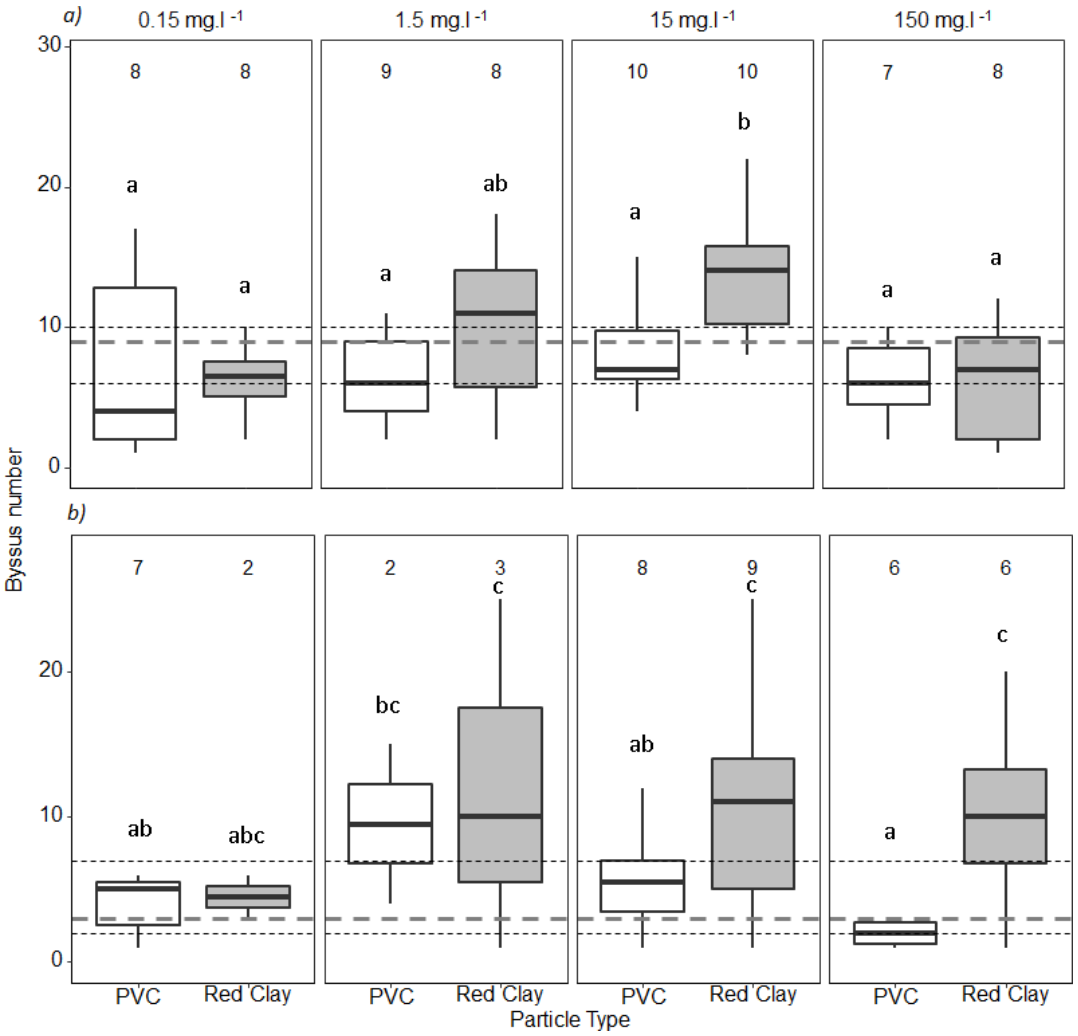

Figure 5. *Mytilus galloprovincialis* byssus numbers per individual after a) three and b) six weeks of particle exposure for each particle type and concentration level. Thick grey dashed line = Median respiration rate of control group (group without particle exposure), thin black lines= lower and upper quartile of control. The number above each bar indicates the number of replicates (N). *Post-hoc* test results are represented by letters above bars, with significant differences indicated by different letters.

Clearance rate

No significant differences were found between the clearance rates of mussels exposed to PVC and RC, neither were any concentration effects detected (Table 2). Average clearance rates of mussels that were exposed to PVC were, however, consistently greater (mean ± SD = 0.55 ± 0.67 ml min $^{-1}$ g^{-1} WW) than those exposed to red clay (mean ± SD = 0.36 ± 0.64 ml min $^{-1}$ g^{-1} WW) (Figure 6). Clearance rates of mussels that were exposed to the lowest PVC concentration were markedly higher than in all other groups, followed by the second highest concentration of PVC.

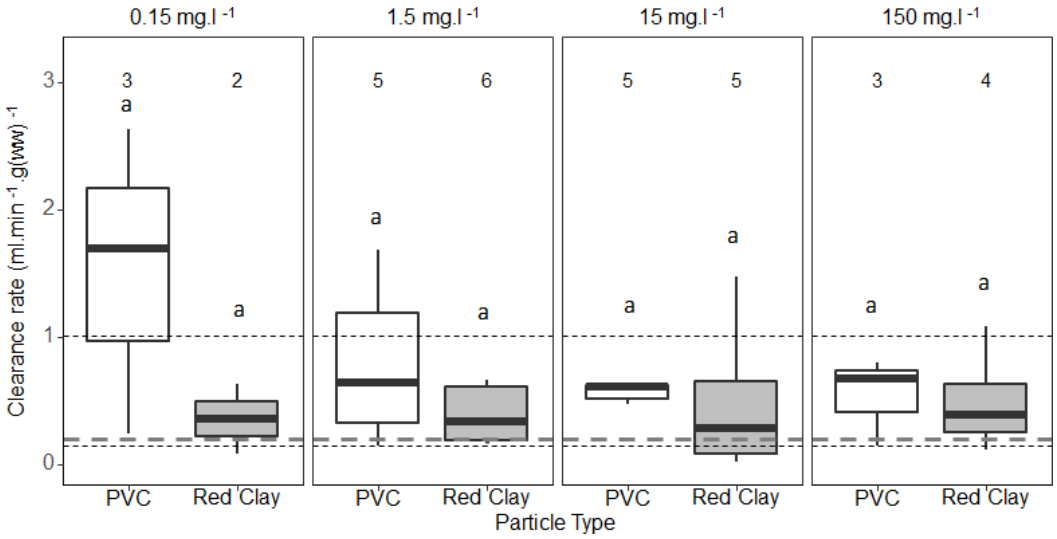

Figure 6. *Mytilus galloprovincialis* clearance rates of individuals after three weeks of particle exposure for each particle type and concentration level. Thick grey dashed line = Median respiration rate of control group (group without particle exposure), thin black lines= lower and upper quartile of control group. The number above each bar indicates the number of replicates (n). *Post-hoc* test results are represented by letters above bars, with significant differences indicated by different letters.

Body Condition Index (BCI)

No effects of particle type and concentration were found on BCI after six weeks of exposure, however a significant interaction between particle type and concentration was found (Table 1). *Post-hoc* tests revealed that all significant results that were detected, were between the high BCI values in the lowest concentration of PVC, and the other groups (Figure 7). Individuals in the lowest PVC concentration had much higher BCI values than the other groups but, the superior health of mussels in this group is likely to be a random effect. All groups, with the exception of the lowest PVC concentration, had BCI close to, or within, the interquartile range of the control group (Figure 7).

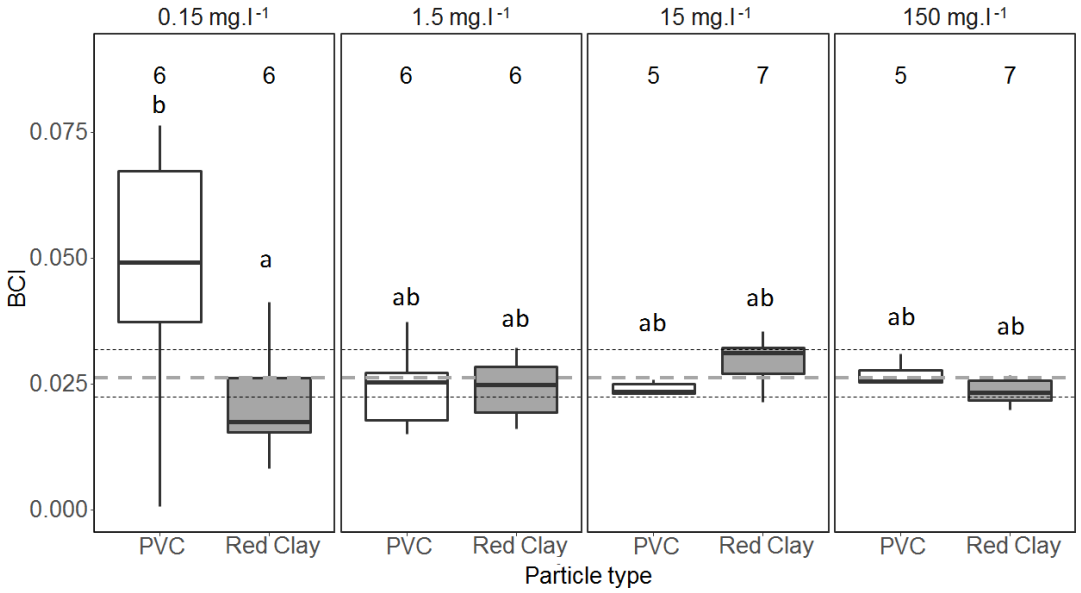

Figure 7. *Mytilus galloprovincialis* Body Condition Index(BCI) of individuals after six weeks of particle exposure for each particle type and concentration level. Thick grey dashed line = Median respiration rate of control group (group without particle exposure), thin black lines= lower and upper quartile of control group. The number above each bar indicates the number of replicates (N). *Post-hoc* test results are represented by letters above bars, with significant differences indicated by different letters.

Survival

A total of 29 mussels died during the experiment, and 89.7% of the mortality events occurred after Day 40. In the PVC treatments, 17 mortality events were recorded, compared to 12 in the red clay treatments. This difference was not statistically significant ($n = 80$, $z = -1.284$, $p = 0.199$), despite the survivorship of mussels in PVC treatments always being below those exposed to red clay (Figure 8a.) Fourteen mortality events were recorded in the two lowest concentrations pooled, and 15 in the two highest concentrations, with no differences found between the two groups ($n = 80$, $z = 0.012$, $p = 0.991$) (Figure 8b).

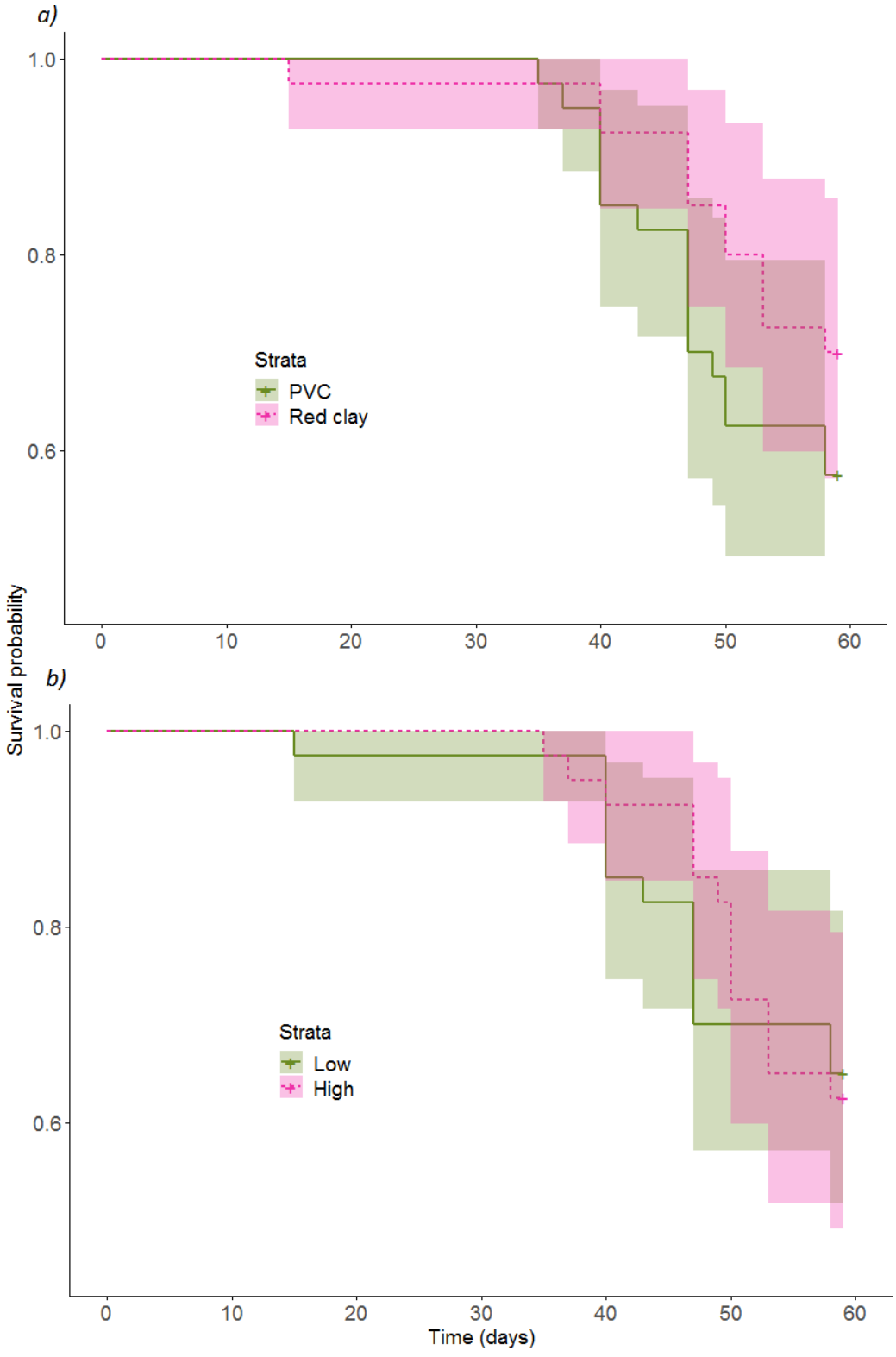

Figure 8. Kaplan-Meier curves comparing the *Mytilus galloprovincialis* survivorship over time (in days) of a) mussels exposed to PVC (green) and red clay (pink) and b) mussels in the two lowest (green) and two highest (pink) concentrations. Shaded areas show the 95% confidence interval of each curve.

3.2. Discussion

The key question in microplastic research in marine systems is whether microplastic particles constitute a novel stressor, and if so to what extent this is significant for the integrity, stability and functioning of marine ecosystems. To properly establish this, it is essential to compare the effects of microplastics to those caused by existing natural stressors. This study aimed to disentangle the effects of microplastic particles from those of naturally occurring particles on mussels, by directly comparing the impacts of the two particle types under the same experimental conditions and, hence, assessing the specific influence of microplastics on marine biota. Overall, the tests mussels were robust to particle exposure, with mussels exposed to the highest concentrations of particles for six weeks showing no difference in their body condition index (BCI) compared to those exposed to no inorganic particles over the same time period. Particle type differences were found in byssus production, but only at high concentrations. Respiration rates were significantly affected by particle concentration, with the highest respiration rates recorded in mussels exposed to the highest particle concentrations, but no particle type effects were found.

After six weeks, mussel respiration rates were significantly affected by particle exposure, with respiration rates increasing with increasing particle concentrations. However, in an almost identical study on *M. galloprovincialis*, no effect of particle concentration or particle type was found over the same concentration range as this study (Yap et al. 2020). Some authors have found that exposure to micro-particles both plastic and natural, reduced respiration rates when exposed to similar particle concentrations as used in this study (Grant and Thorpe 1991, Rist et al. 2016). Grant and Thorpe (1991) found a significant decrease in oxygen consumption when exposing *Mya arenaria* to 100-200 $mg.l^{-1}$ of intertidal sediment. Rist et al. (2016) found a decrease in respiration rate over a larger particle concentration range (0- 2160 $mg.l^{-1}$) in *M. edulis.* Others have speculated about possible compensatory effects, whereby filter feeders maintain a constant respiration rate and absorption efficiency, but reduce clearance rates in order to adjust energy intake in response to obstructions caused by microplastics in the gut (Xu et al. 2017). Widdows et al. (1979) found no linear relationships between respiration rates and natural seston concentrations (2-350 $mg.l^{-1}$) in the closely-related *M. edulis*. Respiration rates have

also been found to increase with increasing sediment concentrations in a variety of filter-feeding taxa. A study on three Antarctic ascidians found that oxygen consumption increased over a concentration range of suspended sediment that was similar to that used in this study and reached maximum respiration rates at sediment concentrations of 100 - 200 mg/L, after which respiration rates slowly decreased (Torre et al. 2012). The increase in oxygen consumption with increased sediment concentration was attributed to increased metabolic costs due to enhanced ciliary action, mucous production and squirting during excretion. Similarly, a study testing the effects of particle concentration on respiration in the freshwater mussel *Dreissena polymorpha* (Pallas, 1771) found that the highest respiration rates were recorded in the highest particle concentration level (100 $mg.l^{-1}$), and attributed this to the energetic costs associated with increased pseudofeces production (Madon et al. 1998). Although pseudofeces werenot quantified in this study, mussels were observed producing psuedofeces when exposed to both particle types, and it is plausible that high particle loads resulted in increased pseudofeces production, which in turn increased energy consumption and respiration rates.

The strongest effect of particle type was found in byssus production after six weeks of exposure. However, differences between particle types were only found at the two highest concentrations, while the picture at the lower concentrations was less clear, which may have been caused by the low replicate numbers due to high proportion of unattached individuals in these groups. Since mussels that failed to produce byssus, were excluded from the analysis, this resulted in low replicate numbers. These low attachment rates, however, were likely to be a random occurrence, as there was no indication that the mussels in these groups were in poorer health. There are studies which have found reduced byssus numbers due to microplastic exposure (Green et al. 2019, Rist et al. 2016), but no effects were found by Yap et al. (2020) when comparing the byssus production of *M. galloprovincialis* exposed to the same concentration levels of PVC and red clay. This, together with the fact that reduced byssus numbers in this study were only found at the particle highest concentrations, is a further indication of the robustness of mussels to exposure to different particle types. The potential mechanism by which byssal production is reduced in the presence of microplastics has not been established. Green *et al.* (2019) did find that microplastics can alter proteins in mussels, including structural proteins, which would likely influence byssal

production. Others have found that microplastics actually become fused within the byssal threads (Li et al. 2019). The fusion of microplastics in byssus is, however, unlikely to influence byssus number, as microplastic particles are thought to adhere to the surface of newly-formed byssus, rather weakening threads than reducing their numbers. A more likely explanation for the observed patterns in this study, is a reliance of mussels on the presence of minerals for the production of byssus (Winkle 1970). Furthermore, studies analysing the chemical composition of byssus threads have found a number of trace metals, which have been proposed to be absorbed through the tissue, rather than adhering to the surface of byssus threads (Coombs and Keller 1981, Romero-Freire et al. 2020). The red clay used has both calcium and magnesium in the form of MgO (3.40%) and CaO (1.30%), which have been shown necessary by Winkle (1970) for the formation of byssal threads (Source of chemical composition of red clay: Argiles du Bassin Méditérranéen®, technical file). Furthermore, studies on the chemical composition of byssus threads have found that those produced by *M. galloprovincialis* have high iron (Fe) and aluminium (Al) content, both of which make up a large proportion of the composition of red clay (Fe_2O_3 = 7.40% and Al_2O_3 = 18.81%, Source of chemical composition of red clay: Argiles du Bassin Méditérranéen®, technical file). Thus, higher concentrations of these elements in red clay treatments may result in higher byssus production. Future investigation comparing the effects of microplastic and natural particles should take this into account and consider finding a natural reference particle that does not affect the availability of minerals and trace elements. No effects of particle type were found in the clearance rates of exposed mussels. This is contrary to Harris and Carrington (2020), who found differences in the clearance rates of mussels exposed to natural and plastic microparticles. The methods used to measure clearance rates in this study were rather similar to those used by Harris and Carrington (2020), but a few differences in methodology may explain the disparity in results. Harris and Carrington (2020) measured clearance rates in the presence of particles, and thus as a direct response to particle exposure, while this study measured carry-over-effects and the measurements were taken in the absence of inorganic particles. Mussels have been shown to adjust clearance rates depending on ambient particle concentrations, although the relationship is unclear and thought to be largely dependent on the particle concentrations used (Griffiths and Griffiths 1987). In this study, measurements were taken in the absence of particles; hence mussels were unlikely to have modified their clearance rates, which explains why no

effects were found. Carry-over effects are meaningful, as they show more permanent effects of mussels, rather than temporary adjustments to changes in environmental conditions. Harris and Carrington (2020) used similar particle concentrations, but of polyethylene (PE) spheres as the microplastic, and silt as the natural particle, making results difficult to compare. Rist et al. (2016) found a significant decrease in clearance rate with particle concentration, but used far higher microplastic concentrations (0 – 2160 $mg.l^{-1}$) than this study. A recent study measuring the carry-over effects of more environmentally relevant microplastic concentrations (0.008 $\mu g\ l^{-1}$, 10 $\mu g\ l^{-1}$, and 100 $\mu g\ l^{-1}$) found no effects on the clearance rates of *M. edulis* (Revel et al. (2019). The measurement of clearance rates has a large variance, even with the use of a flow cytometer, and more replicates would be required to obtain more satisfactory insights. Long exposures such as this should start with greater replicate numbers to account for mortality and poor mussel health at the end of the experiment. It, however, appears that mussels are largely unaffected by microplastic particles, even when exposed to concentrations far greater than those found currently in the ocean.

Mortality itself is a useful indicator of the health of mussels exposed to the different treatments. Survival analysis revealed no significant effects of concentration and particle type. Surprisingly the overall mortality between the two highest and two lowest concentrations pooled, only differed by one event. There is little evidence that high particle concentrations result in higher mortality in mussels, which confirms how well- adapted filter feeding bivalves are at surviving high particle densities. Rahim et al. (2019) found a significant increase in mussel mortality with particle concentration, but the microplastic concentrations used were very high (up to 5 $g.l^{-1}$). The picture is slightly different regarding the effect of particle type, with consistently higher mortality suffered under PVC exposure than red clay. The survivorship curve of mussels exposed to PVC was almost always below that of individuals exposed to red clay. The difference was, however, not statistically significant and hence must be interpreted with caution. Higher mortality in PVC treatments may once again be a result of poorer nutrition due to the absence of required minerals, such as magnesium and calcium, which apart from reducing byssus, also reduce the rate of other physiological functions (Winkle 1970). This may also explain why other authors have failed to find notable or clear effects of microplastics on mussel mortality, even at very high

particle concentrations (Rist et al. 2016, Pedersen et al. 2020). Rist et al (2016) did find significant effects of PVC exposure on the mortality of mussels, although this was only evident at very high concentrations (2160 $mg.l^{-1}$), while Pedersen et al (2020) found no effects on mortality, even at high concentrations of PE (800 $mg.l^{-1}$). Yap et al. (2020) found no effects on mortality using the same particle types and concentration on M. galloprovincialis. Significant effects on mortality have been found in other groups of filter feeders, with a study on *D. magna* finding increased mortality when exposed to 12.5-100 $mg.l^{-1}$ of microplastic fibres (Jemec et al. 2016). The cause of death was proposed to be a result of the clogging of filtering apparatus and gut, rather than leachates from the plastics.

The metabolic pathways through which filter feeders modify their behaviour in the presence of particles are poorly understood and it is thus difficult to speculate about possible drivers for particle type differences. This study found some significant particle type differences, but these were possibly caused by poor nutrition in the PVC treatments, reflecting non-environmentally relevant conditions. The lower concentrations used in this study may be reached in highly industrialized coastal areas in the future, and so far no studies have found severe detrimental effects of microplastic particles on organisms exposed to these concentrations. This work, therefore, adds to a growing body of literature suggesting that microplastic particles do not pose an imminent ecological threat on mussels. This study highlights the importance of pursuing long-term exposures comparing anthropogenic and natural particles in order to improve our understanding of the potential impacts of microplastics on biota, as well as their wider ecological consequences in the marine environment.

4. Experiment 2: Comparative effects of microplastic and natural particles on the physiology of the mussels *Mytilus galloprovincialis* (Lamarck, 1819) and *Choromytilus meridionalis* (Krauss, 1848)

The Results and Discussion outlined in this chapter build upon the Introduction and Methods described in Chapters 1 and 2 above.

4.1. Results

Species comparison of baseline measurements and mortality

The comparison of baseline measurements highlighted important physiological differences between the two species. Baseline respiration rates were significantly different (n = 319, t = 8.16, Df = 145.06, $p < 0.001$), with the mean ± SD respiration rate of *M. galloprovincialis* individuals (0.037 ± 0.008 mg O_2 .h $^{-1}$.g $^{-1}$(ww) $^{-1}$, n =159) being lower than that of *C. meridionalis* (0.050 ± 0.011 mg O_2 .h $^{-1}$.g $^{-1}$(ww) $^{-1}$, n = 160). Byssus numbers also differed between the two species (n = 304, W = 3946.5, $p < 0.001$), with *M. galloprovincialis* and *C. meridionalis* producing 3.7 ± 3.1 and 10.2 ± 6.5 (mean ± SD) byssus numbers, respectively. BCI values of *M. galloprovincialis* and *C. meridionalis* were rather similar (0.047 ± 0.011 and 0.055 ± 0.018, respectively), showing that individuals of the same size had similar body condition. The BCI of *M. galloprovincialis* individuals was slightly lower than *C. meridionalis*, but this difference was only mildly significant (n = 44, t = 1.83, Df = 30.63, p = 0.077).

Respiration rate

In line with baseline measurements, Week 3 respiration rates of *M. galloprovincialis* were significantly lower than those of *C. meridionalis* (Figure 9). Mean respiration rates after three weeks of exposure were 0.020 ± 0.008 mg O_2 .h $^{-1}$.g $^{-1}$(ww) $^{-1}$ for *M. galloprovincialis* and 0.050 ± 0.011 mg O_2 .h $^{-1}$.g $^{-1}$(ww) $^{-1}$ for *C. meridionalis.*

Week 3 respiration rates revealed a significant interaction between species and particle type (Table 3). Post-hoc testing showed that *C. meridonalis* was significantly affected by particle type ($p = 0.021$), whilst no such effect was found on the respiration rates of *M. galloprovincialis* ($p = 0.958$) (Figure 9 and Appendix Figure 16). A significant interaction effect also occurred between species and concentration, reflecting that magnitude of the differences in oxygen consumption between the two species varied among particle concentration treatments (Table 3). A mildly significant effect of particle type was found on the combined respiration rates of *M. galloprovincialis* and *C. meridionalis* (Table 3), although the difference in the mean respiration rates between the two particle types was very small, with a mean ± SD respiration rate of 0.036 ± 0.020 mg O_2 .h^{-1}.g^{-1}(ww)$^{-1}$ and 0.034 ± 0.017 mg O_2 .h^{-1}.g^{-1}(ww)$^{-1}$ for PVC and red clay treatments, respectively.

Week 6 respiration rate measurements once again showed significantly lower respiration rates in *M. galloprovincialis* than in *C. meridionalis* with mean ± SD respiration rates of 0.033 ± 0.011 mg O_2 .h^{-1}.g^{-1}(ww)$^{-1}$ and 0.011 ± 0.006 mg O_2 .h^{-1}.g^{-1}(ww)$^{-1}$, respectively (Figure 10). No other effects were found in the respiration rates after six weeks of exposure (Table 3).

Table 3. Summary of three-way ANOVA results of the effect of particle type, species and concentration and the interactions of the three factors on the respiration rates and BCI of *Choromytilus meridionalis* and *Mytilus galloprovincialis*.

	Sum Sq	Df	F	P
Respiration Week 3				
Species	0.03669	1	399.79	<0.0001***
Particle type	0.00028	1	3.01	0.085
Concentration	0.00009	3	0.34	0.800
Species*Particle type	0.00053	1	5.76	0.018*
Species*Concentration	0.00086	3	3.11	0.029*
Particle type*Concentration	0.00028	3	1.01	0.390
Species*Particle type*Concentration	0.00052	3	1.90	0.133
Respiration Week 6				
Species	0.01698	1	197.86	<0.0001***
Particle type	0.00006	1	0.72	0.397
Concentration	0.00026	3	1.01	0.392
Species*Particle type	0.00023	1	2.74	0.100
Species*Concentration	0.00035	3	1.38	0.252
Particle type*Concentration	0.00008	3	0.30	0.826
Species*Particle type*Concentration	0.00005	3	0.20	0.896
BCI Week 7				
Species	0.08753	1	315.28	<0.0001***
Particle type	0.00004	1	0.15	0.702
Concentration	0.00347	3	4.16	0.008**
Species*Particle type	0.00019	1	0.70	0.406
Species*Concentration	0.00113	3	1.35	0.261
Particle type*Concentration	0.00059	3	0.70	0.552
Species*Particle type*Concentration	0.00188	3	2.26	0.086

Table 4. Summary of GLM results of the effect of particle type, species concentration and their interaction on byssus number and clearance rates of *Choromytilus meridionalis* and *Mytilus galloprovincialis*.

	Chisq	Df	P
Byssus number week 3			
Species	0.3040	1	0.581
Particle type	1.4649	1	0.226
Concentration	8.1415	3	0.043*
Species*Particle type	1.2232	1	0.269
Species*Concentration	7.7951	3	0.050*
Particle type*Concentration	3.3468	3	0.341
Species*Particle type*Concentration	1.2417	3	0.743
Byssus number week 6			
Species	3.2600	1	0.071
Particle type	3.9916	1	0.046*
Concentration	0.6697	3	0.880
Species*Particle type	3.4687	1	0.063
Species*Concentration	3.3769	3	0.337
Particle type*Concentration	2.1110	3	0.550
Species*Particle type*Concentration	3.6763	3	0.300
Clearance rate week 3			
Species	0.1424	1	0.706
Particle type	0.3161	1	0.574
Concentration	2.5754	3	0.462
Species*Particle type	0.0035	1	0.953
Species*Concentration	5.8782	3	0.118
Particle type*Concentration	3.4936	3	0.322
Species*Particle type*Concentration	2.5600	3	0.465

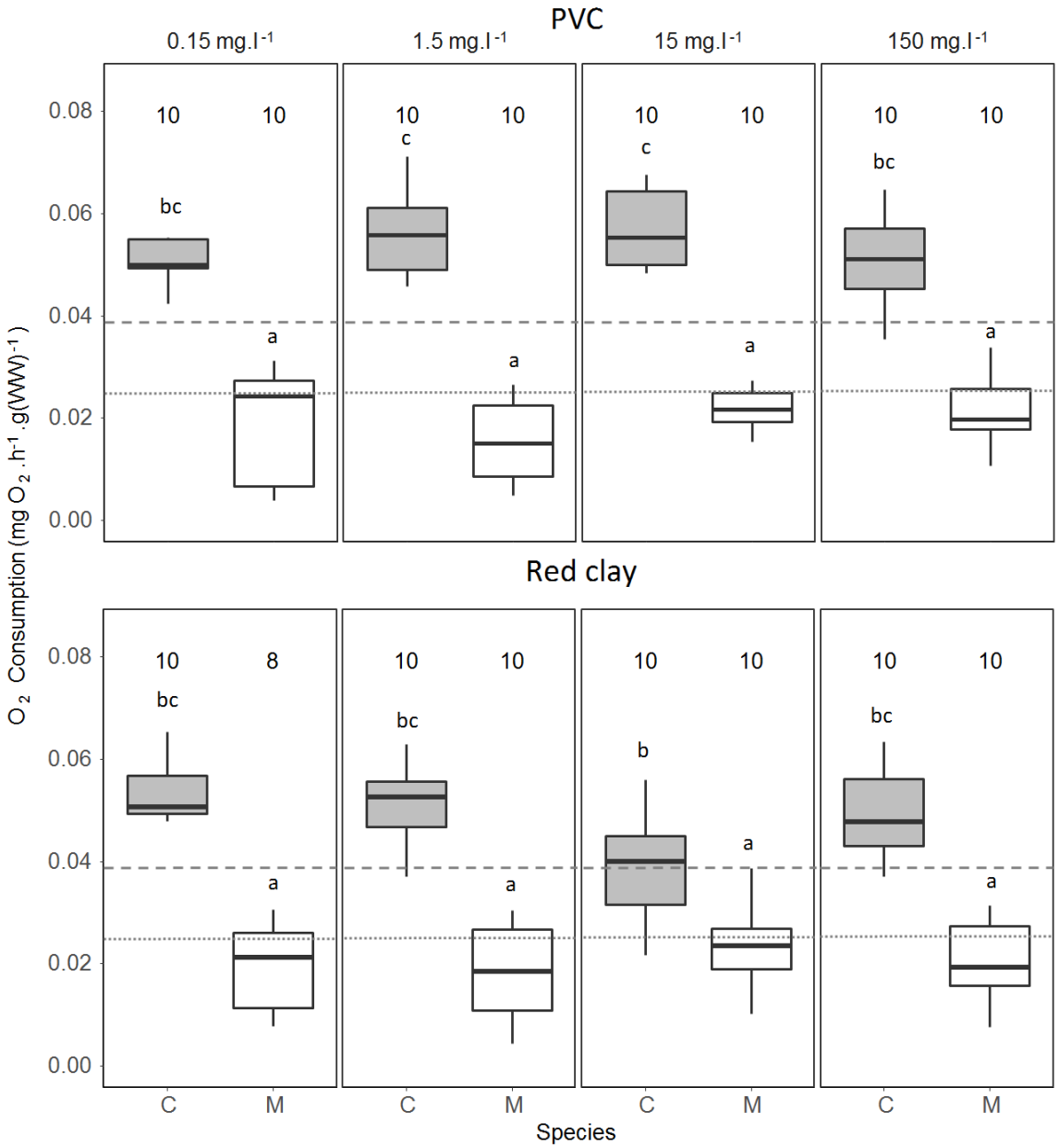

Figure 9. Week 3 respiration rates for the native mussel *Choromytilus meridionalis* (C) and the invasive mussel *Mytilus galloprovincialis* (M) at four different particle concentrations of red clay and PVC. The number above each bar indicates the number of replicates (N). *Post-hoc* test results are represented by letters above bars, with significant differences indicated by different letters. Finely and coarsely dashed lines indicate the median of the control groups of *Mytilus galloprovincialis* and *Choromytilus meridionalis* respectively.

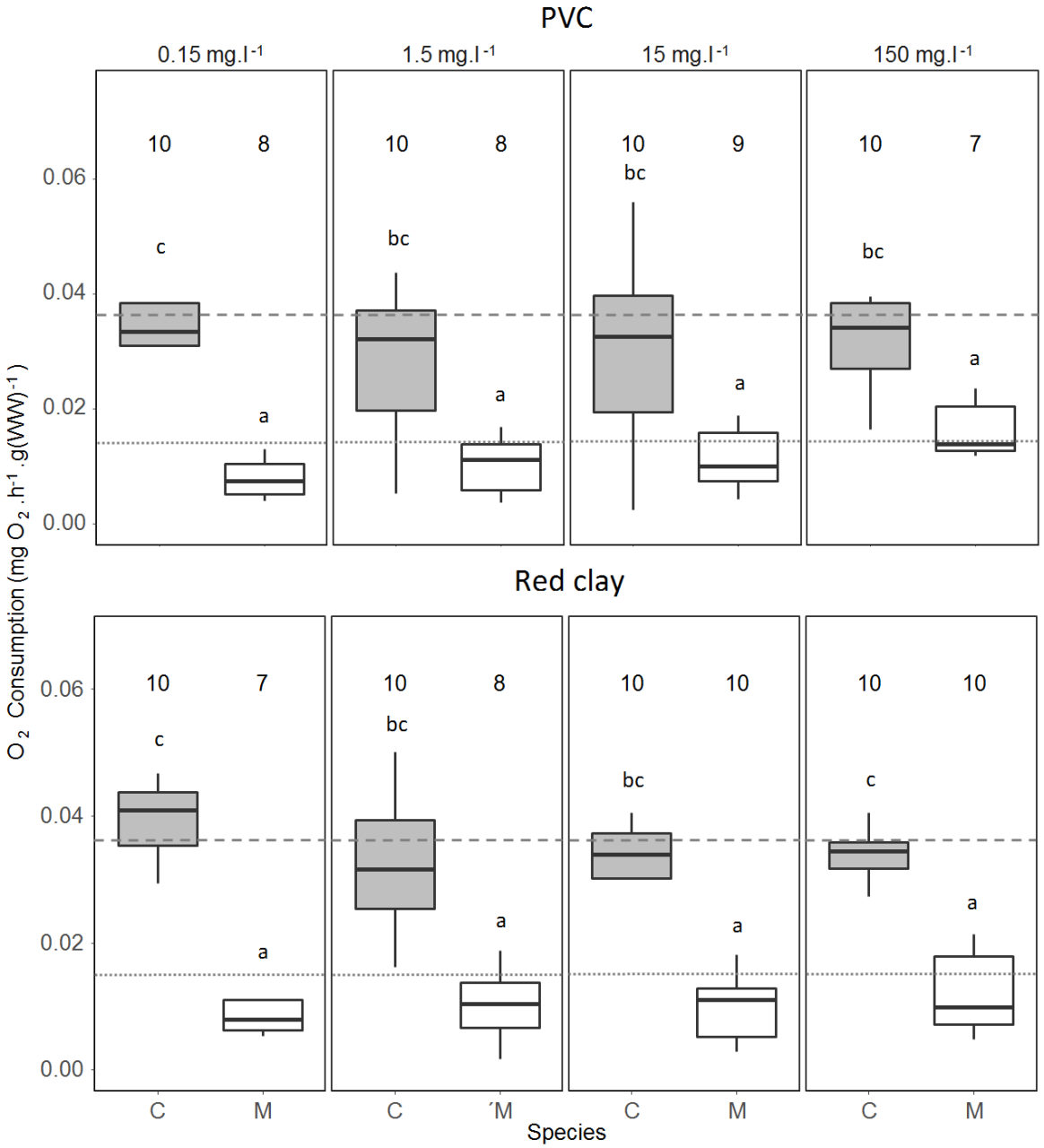

Figure 10. Week 6 respiration rates for the native mussel *Choromytilus meridionalis* (C) and the invasive mussel *Mytilus galloprovincialis* (M) at four different particle concentrations of red clay and PVC. The number above each bar indicates the number of replicates (N). *Post-hoc* test results are represented by letters above bars, with significant differences indicated by different letters. Finely and coarsely dashed lines indicate the median of the control groups of *Mytilus galloprovincialis* and *Choromytilus meridionalis* respectively.

Byssus number

Byssus numbers in Week 3 were similar between the two species, with *M. galloprovincialis* and *C. meridionalis* individuals producing mean ± SD byssus numbers of 8.5 ± 5.6 and 7.9 ± 4.4, respectively (Figure 11). This is despite baseline measurements of byssus numbers showing significant differences between the byssal production of the two species. After three weeks, a significant interaction was found between species and concentration (Table 4). *Post-hoc* tests indicate that notable differences were found between the byssus numbers of the two species at the 15 $mg.l^{-1}$ concentration level ($p = 0.085$), while no differences were found between the byssus numbers in either the lowest or highest concentrations ($p = 0.998$ and 0.997 respectively) (Figure 11).

Week 6 byssus numbers were once again rather similar, with *M. galloprovincialis* (mean ± SD = 7.5 ± 6.3) producing slightly higher byssus numbers than *C. meridionalis* (mean ± SD = 6.3 ± 4.2, although this difference was only mildly significant ($p = 0.071$). A mildly significant interaction between particle type and species was noted, with *M. galloprovincialis* producing fewer byssus in PVC than in red clay treatments ($p = 0.016$), while *C. meridionalis* was unaffected by particle type ($p= 0.967$) (Table 4 and Figure 12). Byssus production in the two species was significantly affected by particle type, which was driven by particle type differences found in *M. galloprovincialis* (Table 4).

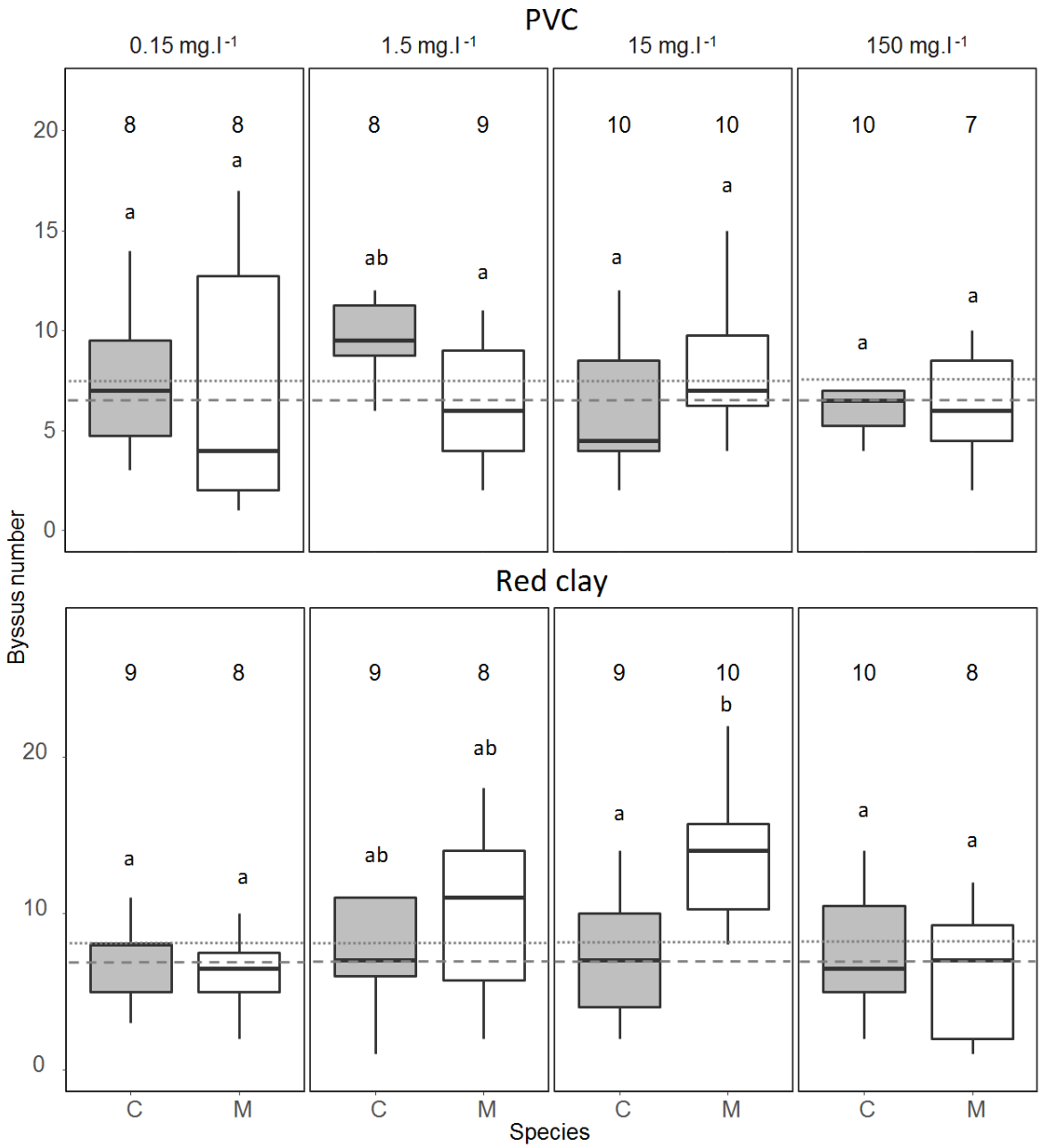

Figure 11. Week 3 byssus numbers for the native mussel *Choromytilus meridionalis* (C) and the invasive mussel *Mytilus galloprovincialis* (M) at four different particle concentrations of red clay and PVC. The number above each bar indicates the number of replicates (N). *Post-hoc* test results are represented by letters above bars, with significant differences indicated by different letters. Finely and coarsely dashed lines indicate the median of the control groups of *Mytilus galloprovincialis* and *Choromytilus meridionalis* respectively.

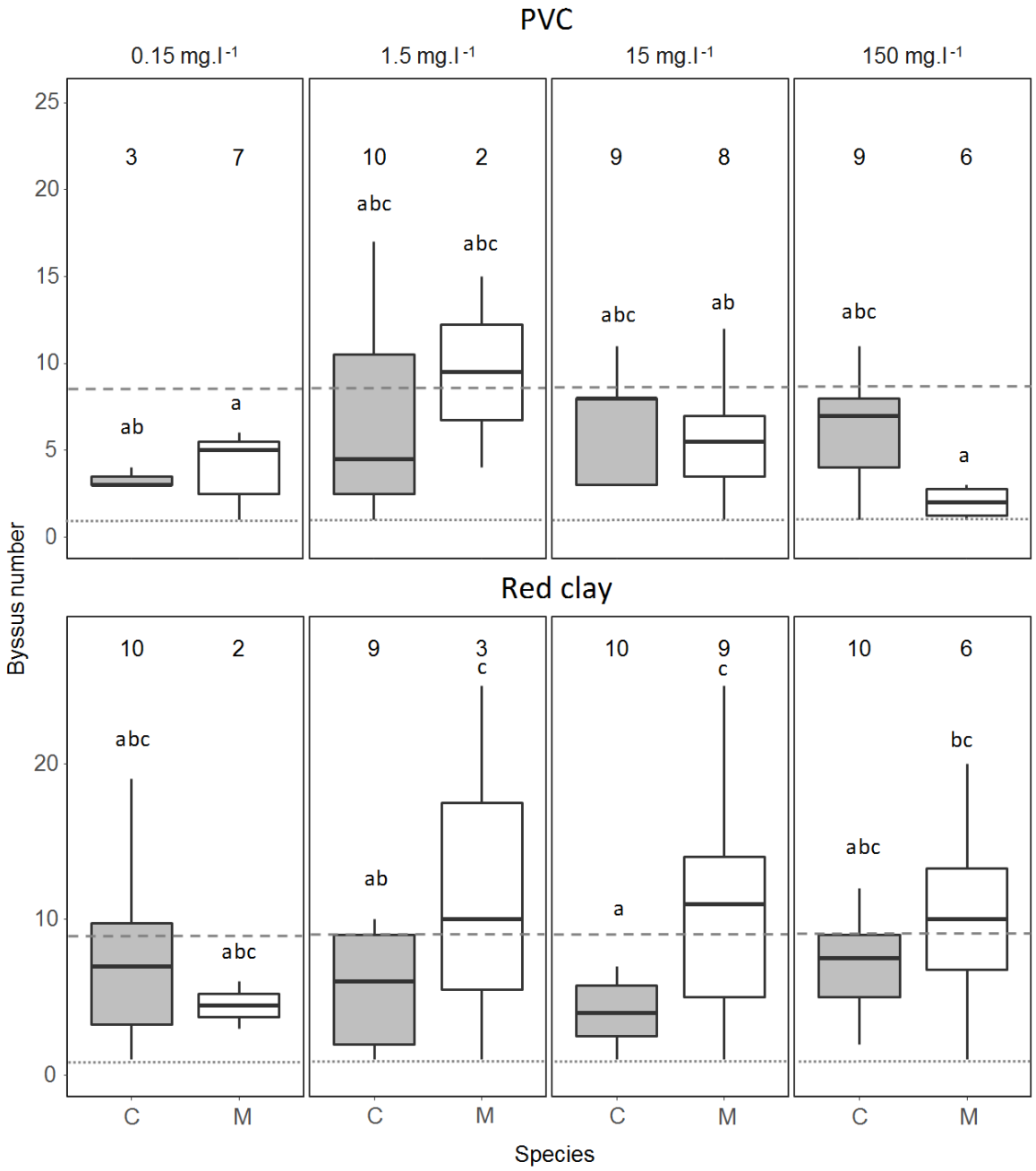

Figure 12. Week 6 byssus numbers for the native mussel *Choromytilus meridionalis* (C) and the invasive mussel *Mytilus galloprovincialis* (M) at four different particle concentrations of red clay and PVC. The number above each bar indicates the number of replicates (N). *Post-hoc* test results are represented by letters above bars, with significant differences indicated by different letters. Finely and coarsely dashed lines indicate the median of the control groups of *Mytilus galloprovincialis* and *Choromytilus meridionalis* respectively.

Clearance rate

The mean clearance rates of the two species were very similar, at 0.7 ± 0.7 ml.min $^{-1}$.g(WW) $^{-1}$ and 0.8 ± 0.1 ml.min $^{-1}$.g(WW) $^{-1}$ (mean ± SD) for *M. galloprovincialis* and *C. meridionalis* respectively. No significant effects were found on the clearance rates of the two species (Table 4). The clearance rates of *C. meridionalis* were consistently higher than for *M. galloprovincialis* in the highest particle concentration treatment, while the opposite was true for the two lowest concentrations of PVC (Figure 13).

Body Condition Index (BCI)

By Week 7, the overall health of *M. galloprovincialis* (mean ± SD BCI = 0.028 ± 0.014) was much poorer than that of *C. meridionalis* (mean ± SD BCI = 0.090 ± 0.051.) (Figure 14). In addition to the significant difference between the BCI of the two species, the body condition of both species was significantly affected by particle concentration (Table 3). *Post-hoc* tests revealed this effect was driven by differences in BCI between the highest and lowest concentrations in both species exposed to PVC treatments, and just in *C. meridionalis* in red clay ones (Figure 14).

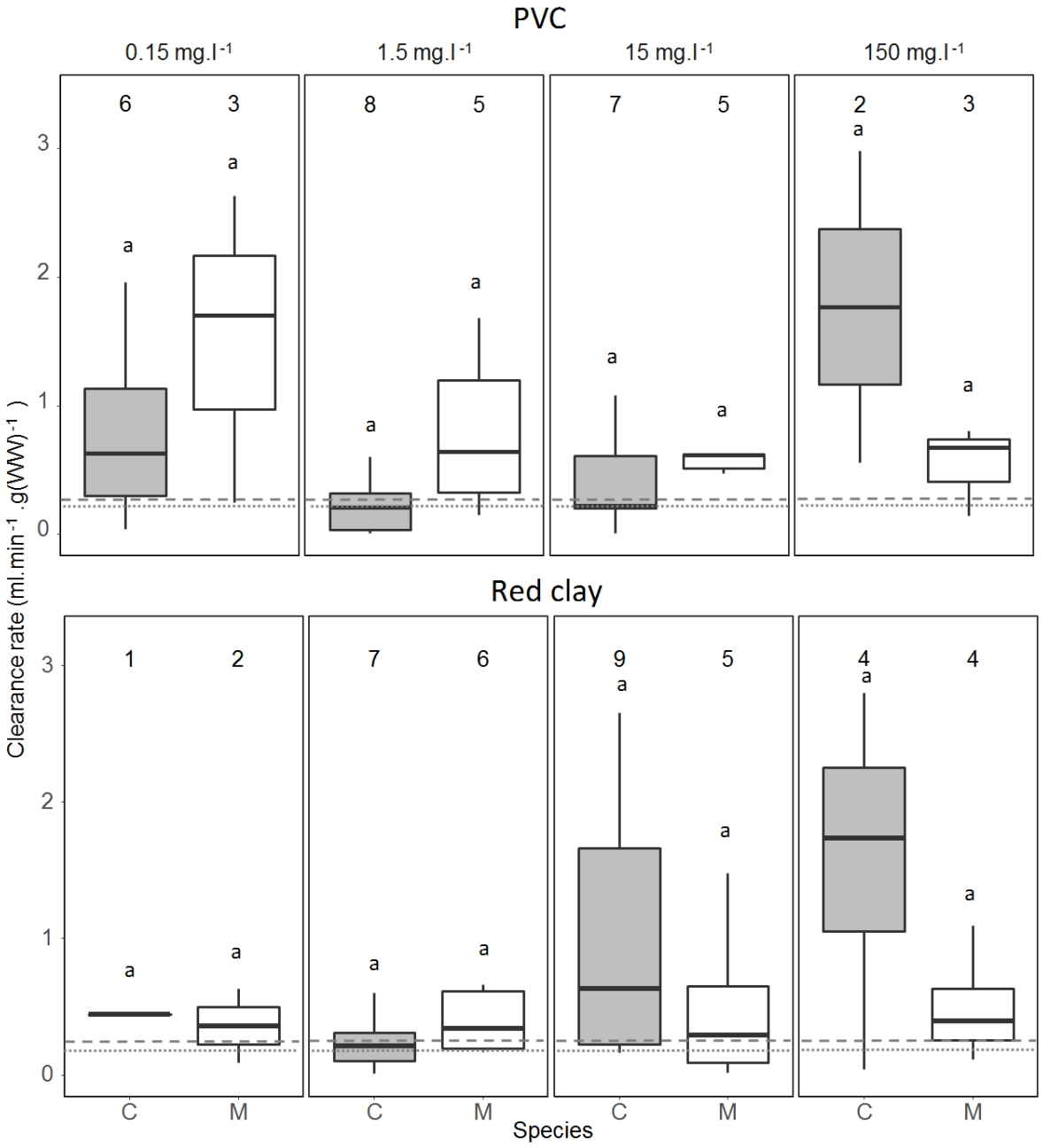

Figure 13. Week 3 clearance rates for the native mussel *Choromytilus meridionalis* (C) and the invasive mussel *Mytilus galloprovincialis* (M) at four different particle concentrations of red clay and PVC. The number above each bar indicates the number of replicates (N). *Post-hoc* test results are represented by letters above bars, with significant differences indicated by different letters. Finely and coarsely dashed lines indicate the median of the control groups of *Mytilus galloprovincialis* and *Choromytilus meridionalis* respectively.

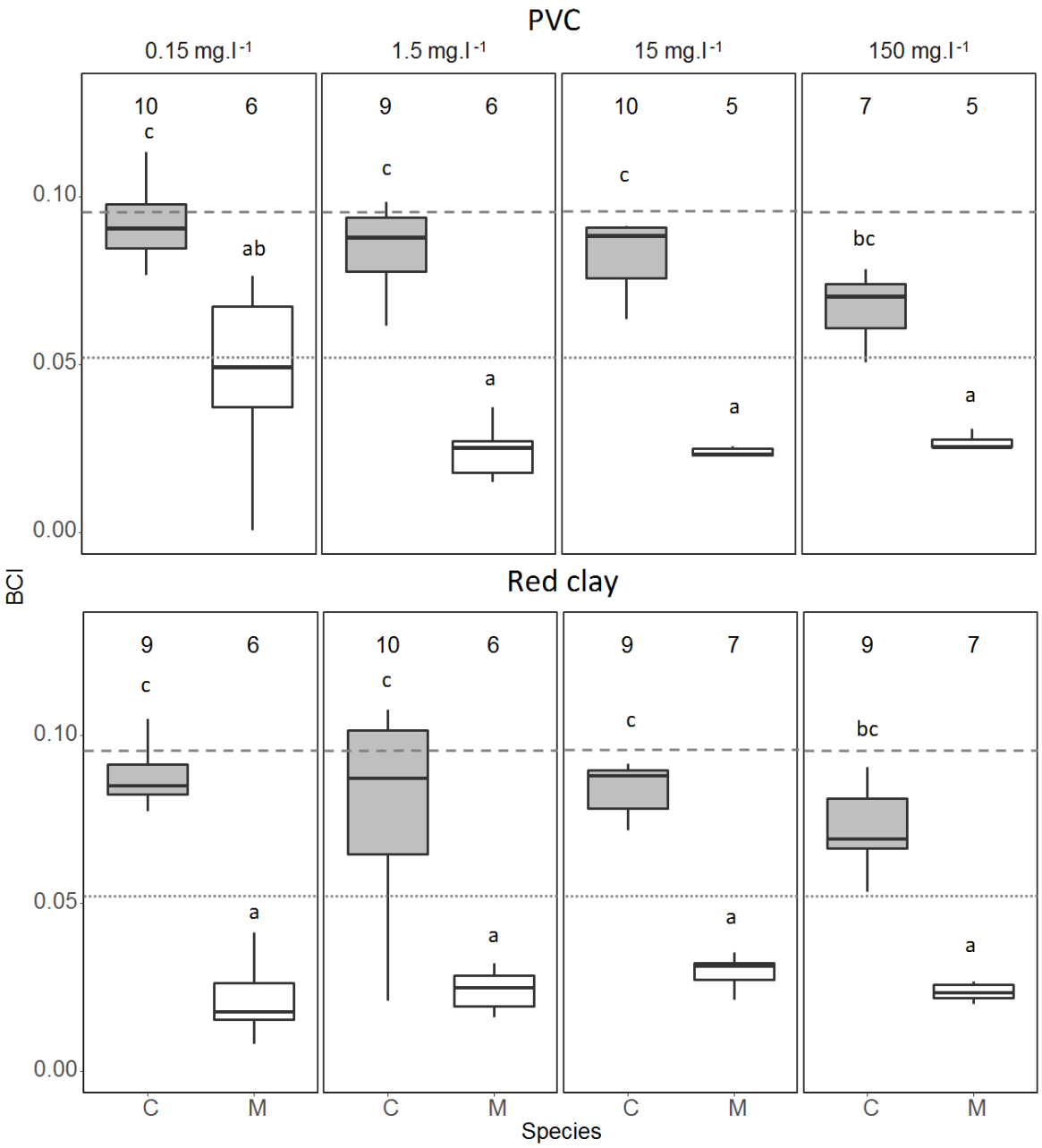

Figure 14. Week 7 Body Condition Index (BCI) for the native mussel *Choromytilus meridionalis* (C) and the invasive mussel *Mytilus galloprovincialis* (M) at four different particle concentrations of red clay and PVC. The number above each bar indicates the number of replicates (N). *Post-hoc* test results are represented by letters above bars, with significant differences indicated by different letters. Finely and coarsely dashed lines indicate the median of the control groups of *Mytilus galloprovincialis* and *Choromytilus meridionalis* respectively.

4.2. Discussion

Sediment has an important influence in shaping intertidal communities and is a significant driver in maintaining a balance between species that are tolerant to sediment and those that are intolerant (Taylor and Littler, 1982). Since the invasion of *M. galloprovincialis*, a number of drastic ecosystem changes have occurred along the South African coast. Slow growing mussel species, such as *Aulocomya atra,* have been largely displaced on the low shore, whilst *C. meridionalis* has been partially displaced, and now thrives mostly in heavily-silted refuges (Robinson et al., 2007). The ability of *C. meridionalis* to adapt to highly silted conditions is likely a key factor in the habitat differentiation (segregation) of *C. meridionalis* and *M. galloprovincialis*, with the latter being confined to areas not prone to sand inundation.

This study aimed to compare the effects of particle exposure on key physiological parameters in *M. galloprovincialis* and *C. merdionalis,* to assess whether different physiological responses to suspended particles may have contributed towards the ability of the two species to coexist via habitat segregation. Physiological differences between the two species were confirmed by the analysis of the baseline measurements, which highlighted lower respiration rates and byssus numbers in *M. galloprovincialis* compared to *C. meridionalis*. The differences in the physiology of these two species were further established by the contrasting effects of the two particle types on byssus production, with *M. galloprovincialis* showing reduced byssus production when exposed to PVC, while no particle type effects were found in *C. meridionalis*. Respiration rates, byssus numbers, clearance rates and BCI also showed different responses in the two species to particle concentration. The responses by both species to particle concentration and particle type were however not very strong, and the differences between species were far more pronounced than the different responses towards particle type and particle concentration.

The most striking difference between the two species was their respiration rates. Throughout the experiment, *M. galloprovincialis* had much lower oxygen consumption rates than *C. meridionalis*, including in baseline measurements. This indicates that the *M. galloprovincialis* individuals collected had a slower metabolic rate than *C. meridionalis,* irrespective of particle concentration, particle type,

or measurement event. A comparison of the respiration rates of four mussel species along the South African coast confirmed *C. meridionalis* respiration rates were generally faster than those of *M. galloprovincialis* (van Erkom Schurink, 1992). However, the extent of the difference in this study indicates that perhaps the *M. galloprovincialis* individuals collected may have had a poorer overall body condition. *Mytilus galloprovincialis* individuals had lower baseline BCI values than *C. meridionalis*, which confirms a disparity in the body condition of the two species prior to the commencement of the experiment.

The respiration rates of the two mussel species also responded differently to particle concentration. The significant interaction between species and concentration was, however, only detected in respiration rates after three weeks of particle exposure and was absent after six weeks. This was likely due to the small difference in respiration rates among individual concentration levels of each species, and thus ANOVA could not detect the overall effect. Differences in the response to particle type were also found between the two species, with *C. meridionalis* showing reduced respiration rates after three weeks of exposure in red clay treatments, while no particle type differences were found in *M. galloprovincialis*. This effect, however, was not robust as it was no longer present, if not reversed, after six weeks of exposure.

Byssus numbers did not show significant differences between species at three and six weeks, despite the significantly higher byssus numbers in *C. meridionalis* documented in the baseline measurements. Byssus production in response to particle type differed in the two species, with *M. galloprovincialis* showing a reduction in byssus numbers in PVC treatments after both three and six weeks of particle exposure, while *C. meridionalis* was unaffected by particle type in both sets of measurements. It is possible that the particle type, and concentration effects on the byssus production of *M. galloprovincialis*, were caused by elements needed for the formation of byssus being only present in red clay (see section 3). The absence of these elements in the PVC treatments, and optimal concentrations of these elements in the middle concentrations of red clay, possibly resulted in augmented byssus numbers in mussels exposed to red clay. Notable variability in the chemical composition of byssus threads have been found across species of mussels, and thus it is reasonable to

speculate that there may be a chemical explanation for the observed species differences (Bouhlel et al., 2017). Considering the significant species differences in byssal thread chemical composition, *M. galloprovincialis* may have a stronger reliance on elements present in red clay treatments, and absent from PVC ones, such as iron (Fe) and aluminium (Al) (Fe_2O_3 = 7.40% and Al_2O_3 = 18.81%, Source of red clay chemical compositions: Argiles du Bassin Méditérranéen®, technical file). These elements make up a considerable proportion of the chemical composition of red clay, as well as of the byssus threads of *M. galloprovincialis* (Bouhlel et al., 2017). The chemical composition of byssal threads of *C. meridionalis* is yet to be analysed, and more clarity could be gained with such information. It, however, appears unlikely that the differences observed in the responses to the particle types between the two species was due to a disparity in the harmful effects of microplastic particles in the two species.

Overall, the clearance rates after three weeks of exposure of the two species were rather similar, with *M. galloprovincialis* exhibiting slightly higher rates than *C. meridionalis*. This is once again supported by the work done by van Erkom Shurink and Griffiths (1992), who found marginally higher clearance rates in *C. meridionalis* than in *M. galloprovincialis*. No significant effects were found for the clearance rates between different treatment levels of the two species, although in *C. meridionalis*, consistently higher clearance rates were observed in the highest concentrations of both PVC and red clay. This reveals a potentially interesting difference in strategy between the two species to increasing particle concentrations, with *M. galloprovincialis* individuals increasing their respiration rates while maintaining a constant clearance rate, while *C. meridionalis* maintained a constant respiration rate and increasing clearance rates. What is curious about the latter result is that most authors assessing the effects of particle concentrations on filtration rates have found that particle concentrations greater than 10 $mg.l^{-1}$ result in decreasing pumping rates (Ali, 1970; Mathers, 1974). It is important to note, however, that the measurements in this study were done in the absence of particles, as the objective was to detect long-term effects of particle concentration, rather than acute reactions to environmental conditions. It is therefore possible that mussels exposed to higher concentrations prior to the measurements had reduced their clearance rates during the exposure time and increased them in response to the presence of water devoid of inorganic particles during the measurements. Respiration rates, however, remained constant

at different particle concentrations, indicating that compensatory responses to the effects of particle exposure in *C. meridionalis* were primarily driven by adjustments in clearance rates. Similar effects have been found in *M. edulis*, with clearance rates increasing until particle concentrations reach approximately 150 $mg.l^{-1}$, and respiration rates remaining constant over the same particle concentration range (Widdows et al., 1979). The reverse was true in *M. galloprovincialis*, where respiration rates were found to increase with particle concentration; this was likely due to individuals at medium and higher concentrations slowing their metabolism during the period of particle exposure prior to the measurement of variables. When placed in clear water, mussels previously exposed to higher concentrations would likely exhibit higher respiration rates to compensate for metabolic losses, possibly augmented by increased psuedofeces production or valve shutting at higher particle concentration. Most research on the responses of these two variables to increasing particle concentrations have been done in the presence of particles, and therefore it is difficult to compare these results to previously published material. The results presented do, however, clearly indicate a different coping strategy in the two species when adapting to varying particle concentration.

The body condition index (BCI) is arguably one of the more useful indicators of overall mussel health, and a significant concentration effect was found on the combined BCI of both species, with *M. galloprovincialis* showing much poorer health than *C. meridionalis*, although this was not linked to the experimental treatment levels. Both species have two spawning seasons over similar times of the year with one in autumn/winter and another in spring/summer (van Erkom Schurink and Griffiths, 1991). According to van Erkom Schurink and Griffiths (1991), declines in body condition associated with spawning usually occur slightly earlier with *M. galloprovincialis* than *C. meridionalis*, and a difference in reproductive stage is the likely have caused the lower BCI values observed throughout the experiment in *M. galloprovincialis*. The overall concentration effect was found to be driven by differences in BCI between the highest and lowest concentrations. The most robust effect was, however, found in *C. meridionalis*, with lower BCI values in the highest concentrations of both PVC and red clay, whereas *M. galloprovincialis* only displayed much higher BCI values in the lowest PVC concentration, while the other treatment levels all showed no differences. Additionally, linear regressions revealed a

significant decrease in BCI with particle concentration in *C. meridionalis*, while no trends were observed in *M. galloprovincialis*. Body Condition Index differences between concentration levels in *C. meridionalis* were only noted at the highest Concentrations. Nevertheless, mussel condition was found to be more strongly affected by particle concentration in *C. meridionalis* than in *M. galloprovincialis*, but species differences were more pronounced than differences in the effects of particle concentrations on the two species.

Given the overall decline in BCI with particle concentration, robustness (resilience) to suspended particle exposure is thus unlikely to be a contributing factor to improved survival of *C. meridionalis* in areas prone to high levels of silt. A much more likely explanation, although unstudied, is a difference in survival rate under periods of anoxic conditions brought about by burial. *Mytilus galloprovincialis* has been found to have improved survival under anoxic conditions when compared to *Perna perna* (Zardi et al., 2006), but has not been compared with *C. meridionalis*. To better understand the underlying physiological determinants for the well-defined habitat segregation observed in *M. galloprovincialis* and *C. meridionalis*, a comparison between their ability to survive anoxic conditions caused by sand inundation should be undertaken.

Experiments such as this study are vital in improving our understanding of the interaction between this now widespread invasive species and native fauna. The establishment of *M. galloprovincialis* as the dominant mussel species along the west and south coast of South Africa has had a profound impact on native mussel species, which have accommodated to this new resident largely by altering their distributions in the intertidal (Branch et al., 2004). Overall, the two species showed considerable physiological differences, but differences in their reactions to particle loads were not strong and thus it is unlikely to have contributed in the habitat segregation observed. An understanding of the different physiological reactions to environmental and anthropogenic stress between *M. galloprovincialis* and native fauna will improve the existing knowledge on its full potential invasion risk and future ecological impact.

5. General Conclusions

The first experimental chapter of this dissertation, which attempted to address one of the inherent flaws in microplastic research, the lack of a control group exposed to natural particles, found, like many such studies, that mussels are very robust to changes in particle exposure. The chapter attempted to disentangle the effects of microplastic particles and natural inorganic particles on the invasive mussel *Mytilus galloprovincialis*. It was hypothesized (i) that exposure to microplastic particles, as well as (ii) increasing particle concentrations, would result in reduced mussel health. Most variables showed no consistent response to the two particles used (PVC and red clay) and only moderate adjustments towards the different concentration levels. Considering the high particle density in the most concentrated suspensions used, this is quite remarkable. The only notable particle type effect found was in byssus production, where reduced byssus numbers were recorded in mussels exposed to PVC. This, however, was more likely a result of higher concentrations of elements which have been shown necessary for the production of byssus being present in red clay treatments. The next most notable effect was the response of increasing respiration rates with particle concentration. The cause of this is unclear, but it may be a result of increased pseudofeces production at high particle concentrations which would result in increased metabolic costs. Mortality was also consistently higher in PVC treatments than in red clay treatments, although this was not statistically significant. Particle concentration effects were found on the respiration rates and byssus production, but neither of these effects indicated a significant loss in mussel vigour. This was further supported by the fact that body condition index and mortality showed no effect of particle concentrations. In conclusion, there is some indication that PVC resulted in more detrimental effects, and this topic should be the focus for future investigation. As the concentration of microplastic particles in the environment continues to rise, the need for clarity on the potential ecological ramifications of this is becoming more pressing.

The second experimental chapter compared the effects of the two particle types (PVC and red clay particles) and particle concentration on two regional mussel species, the invasive *M. galloprovincialis* and indigenous *Choromytilus meridionalis*. The aims of this chapter were to assess whether the two

mussel species responded differently to the particle treatments, and to assess whether there may be a difference in the fitness of the two species when exposed to suspended particles. This might also explain the distinct habitat segregation observed between the two species. The invasion of *M. galloprovincialis* has resulted in its dominance on the rocky shore, and the native *C. meridionalis* is now largely restricted to areas prone to sand inundation and high silt loads. It was hypothesized that (iii) the two species may differ in their responses to the two particle types, and that (iv) *C. meridionalis* might show fewer detrimental effects of increasing concentrations of suspended particles than *M. galloprovincialis*. The findings show no indication that *C. meridionalis* is better suited to dealing with suspended particles, but did reveal notable differences in physiology of the two species. Most strikingly, *M. galloprovincialis* had much lower respiration rates and BCI values than *C. meridionalis* throughout the experiment. More subtle differences to particle loads were noted, with *M. galloprovincialis* showing increased respiration rates and to maintaining a constant clearance rate in response to increasing particle concentrations, while *C. meridionalis* increased clearance rates, and maintained a constant respiration rate. *M. galloprovincialis* byssus numbers were also significantly affected by particle type, while *C. meridionalis* showed no particle type effects. Further research is needed to assess how these different strategies may affect the relative fitness of the two species in environments with high particle loads. The fact that decreasing BCI with particle concentration was found in *C. meridionalis*, while no effect was found on the BCI of *M. galloprovincialis*, suggests that it is unlikely that *C. meridionalis* is more robust to suspended particles.

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7. Appendix

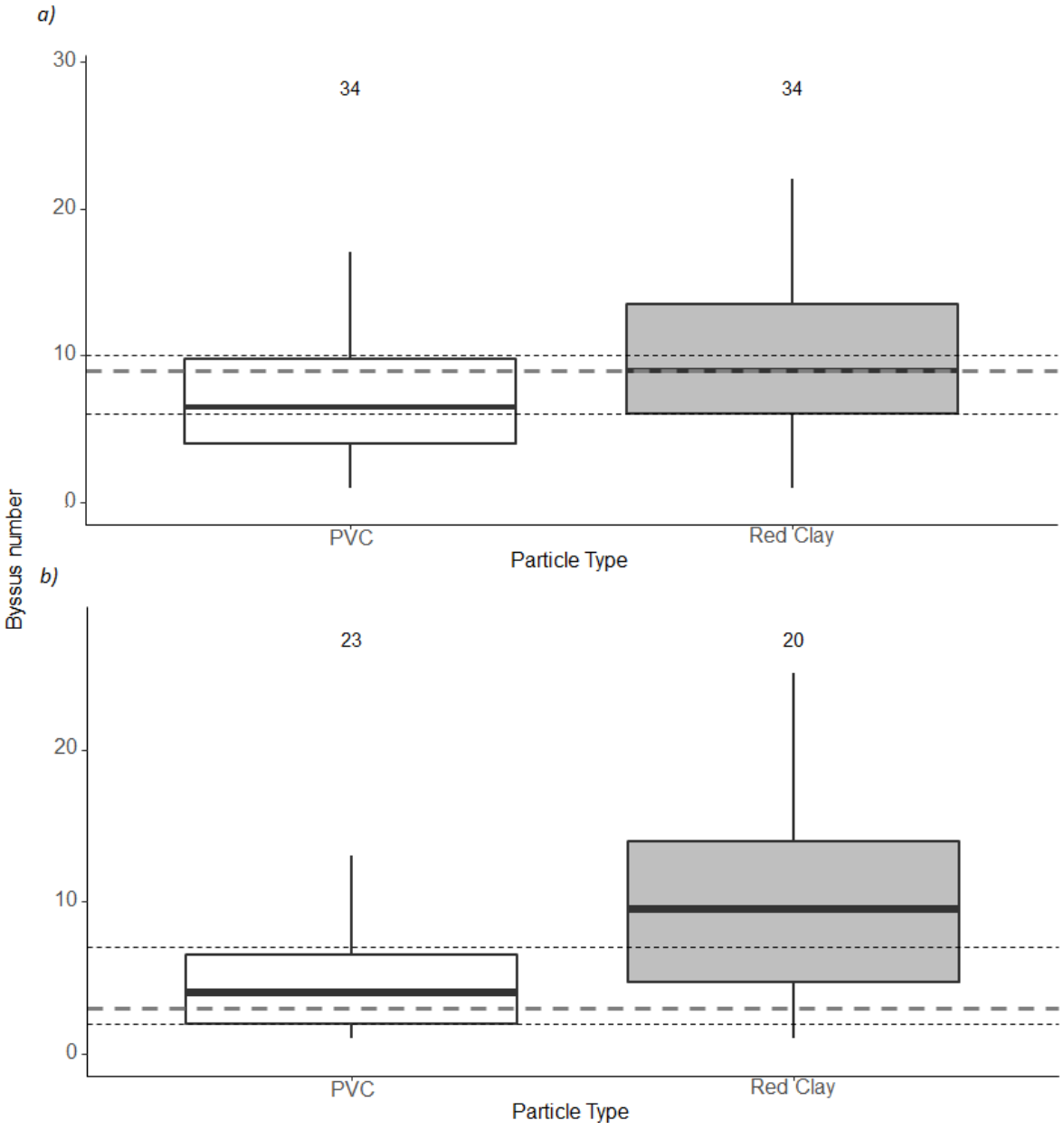

Figure 15. *Mytilus galloprovincialis* byssus numbers per individual after a) three and b) six weeks of particle exposure for each particle type with pooled concentrations. Thick grey dashed line = Median respiration rate of control group (group without particle exposure), thin black lines= lower and upper quartile of control group. The number above each bar indicates the number of replicates (N).

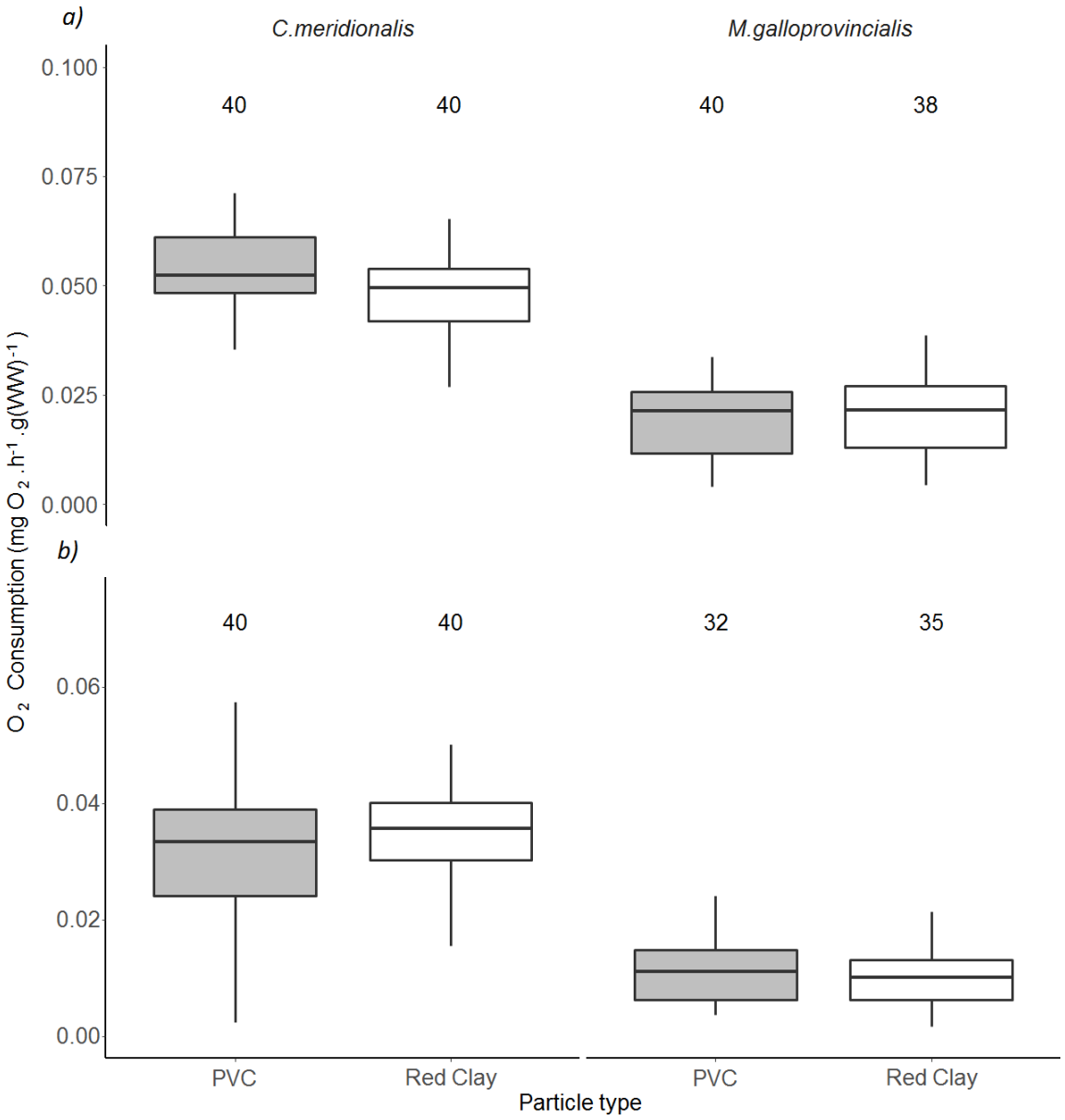

Figure 16. Respiration rates at different particle types for *Choromytilus meridionalis* and *Mytilus galloprovincialis* after a) three and b) six weeks of exposure.

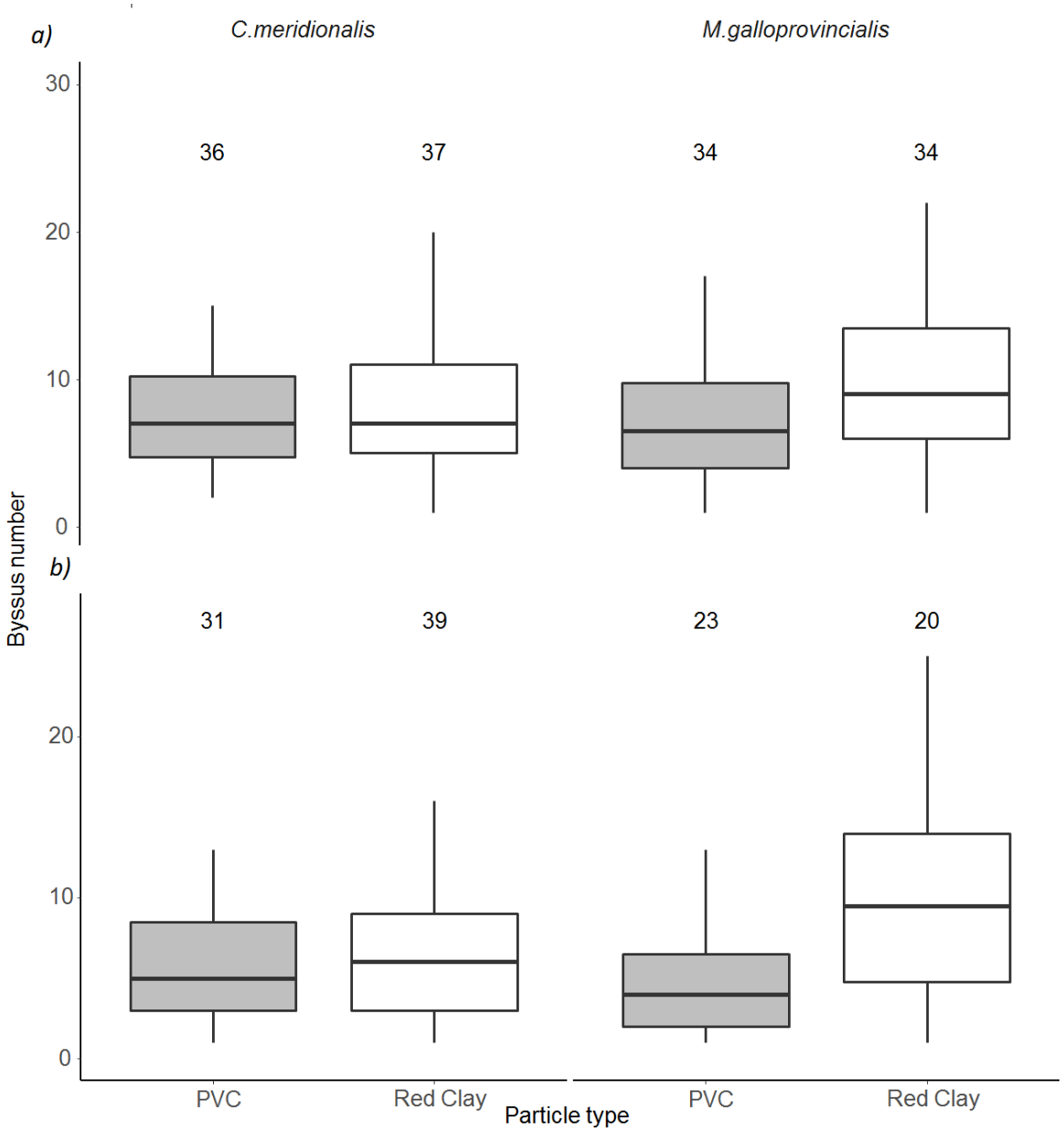

Figure 17. Byssus numbers at different particle types for *Choromytilus meridionalis* and *Mytilus galloprovincialis* after a) three and b) six weeks of exposure.